CUK-H03684-4-P024844

ANNUAL REVIEW OF MEDICINE

④

P, 24 844

EDITORIAL COMMITTEE

C. H. COGGINS
Harvard Medical School

W. P. CREGER
Stanford University

N. FREINKEL
Northwestern University

E. W. HANCOCK
Stanford University

D. C. SABISTON
Duke University

E. J. SACHAR
Albert Einstein College of Medicine

J. P. SANFORD
The University of Texas

R. T. SMITH
The University of Florida

L. W. WANNAMAKER
The University of Minnesota

Responsible for the organization of Volume 25
(Editorial Committee, 1972)

B. CHILDS
Johns Hopkins University

W. P. CREGER
Stanford University

A. C. DEGRAFF
New York University

N. FREINKEL
Northwestern University

S. S. KETY
Harvard Medical School

J. P. SANFORD
The University of Texas

C. A. STETSON JR.
New York University

ANNUAL REVIEW OF MEDICINE

④

WILLIAM P. CREGER, *Editor*
Stanford University School of Medicine

C. H. COGGINS, *Associate Editor*
Harvard Medical School

E. W. HANCOCK, *Associate Editor*
Stanford University School of Medicine

VOLUME 25

1974

ANNUAL REVIEWS INC. 4139 EL CAMINO WAY PALO ALTO, CALIFORNIA 94306

UNIVERSITY COLLEGE OF MEDICINE
LIBRARY
C. U.

616·0058
An 78
V. 25

1523

ANNUAL REVIEWS INC.
Palo Alto, California, USA

COPYRIGHT © 1974 BY ANNUAL REVIEWS INC.
ALL RIGHTS RESERVED

International Standard Book Number 0–8243–0525–6
Library of Congress Catalog Card Number A51–1659

Assistant Editor Joanne Cuthbertson
Compositor Datagraphics Press Inc.

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA

Annual Reviews Inc. and the
Editors of its publications assume
no responsibility for the
statements expressed by the
contributors to this *Review.*

CONTENTS

CARDIOLOGY:

Sulfonylureas and the Heart, *Gerald S. Levey, Kenneth C. Lasseter, and Roger F. Palmer* 69

Diphenylhydantoin as an Antiarrhythmic Drug, *Arthur J. Atkinson and Richard Davison* 99

Antiarrhythmic Actions of Lidocaine, *Donald C. Harrison and Ken A. Collinsworth* 143

Static (Isometric) Exercise and the Heart: Physiological and Clinical Considerations, *Jere H. Mitchell and Kern Wildenthal* 369

ENDOCRINOLOGY:

Isolated Growth Hormone Deficiency and Related Disorders, *Thomas J. Merimee* 137

Serum Triiodothyronine in Man, *Robert D. Utiger* 289

Vasopressin in Osmotic Regulation in Man, *Gary L. Robertson* 315

Vitamin D Metabolism, *M. F. Holick and H. F. DeLuca* 349

GASTROENTEROLOGY:

Role of Gastrin in Hypersecretory Disorders in Man, *James E. McGuigan* 233

GENETICS:

Genetic Aspects of Gout, *Michael A. Becker and J. Edwin Seegmiller* 15

HEMATOLOGY:

2,3-DPG and Erythrocyte Oxygen Affinity, *George J. Brewer* 29

Treatment of Acute Granulocytic Leukemias, *J. Bernard, M. Weil, and Cl. Jacquillat* 39

Mechanism of Action of Hemoglobin, *Arthur Arnone* 123

Immuno-Prevention of Rh Hemolytic Disease of the Newborn, *R. B. McConnell and J. C. Woodrow* 165

Marrow Transplantation for Aplastic Anemia and Acute Leukemia, *P. Neiman, E. D. Thomas, C. D. Buckner, R. Storb, A. Fefer, H. Glucksberg, and K. G. Lerner* 179

Clinical Utility of In Vitro Evaluation of Granulopoiesis, *Peter L. Greenberg and Stanley L. Schrier* 269

Molecular Immunology of Factor VIII, *Theodore S. Zimmerman and Thomas S. Edgington* 303

Control of Platelet Production, *Laurence A. Harker* 383

Intravascular Coagulation in Liver Disease, *M. Verstraete, J. Vermylen, and D. Collen* 447

IMMUNOLOGY:

Animal Models of the Asthmatic State, *Roy Patterson and Joseph F. Kelly* 53

Characterization of Amyloid, *George G. Glenner and William D. Terry* 131
Immunopathogenesis of Systemic Lupus Erythematosus, *David Koffler* 149

INFECTIOUS DISEASE:

Venezuelan Equine Encephalitis Virus Infection in Man, *N. Joel Ehrenkrantz and Arnoldo K. Ventura* 9
Food Poisoning Due to *Vibrio Parahaemolyticus, William H. Barker, Jr. and Eugene J. Gangarosa* 75
Preventive Therapy of Tuberculosis, *Donald L. Brummer* 115
Infectious Complications of Nonalcoholic Drug Abuse, *Donald B. Louria* 219
Antiviral Chemotherapy, *James P. Luby, M. Tracy Johnson, and Stephen R. Jones* 251

MISCELLANEOUS:

Crystal Deposition Joint Disease, *Daniel J. McCarty* 279
Medical Aspects of Recreational Skin and Scuba Diving, *Jay P. Sanford* 401

NEOPLASIA:

Medical Management of the Hypercalcemia of Malignancy, *Leonard J. Deftos and Robert Neer* 323
The Cell Surface and Malignant Transformation, *R. E. Pollack and P. V. C. Hough* 431

NEPHROLOGY:

Immunopathology and Glomerulonephritis, *Curtis B. Wilson and Frank J. Dixon* 83

NUTRITION:

Modification of Brain Serotonin by the Diet, *John D. Fernstrom* 1

PSYCHIATRY:

Pharmacotherapy of Schizophrenia, *Gerald L. Klerman* 199
Biogenic Amines and Affective Disorders, *Joseph J. Schildkraut* 333

TOXICOLOGY:

The Effects of Fluorinated Anesthetics on the Liver and Kidneys, *Leonard S. Gottlieb and Charles Trey* 411

REPRINT INFORMATION 456

CUMULATIVE INDEX OF CONTRIBUTING AUTHORS, VOLUMES 21 TO 25 457

CUMULATIVE INDEX OF CHAPTER TITLES, VOLUMES 21 TO 25 459

Copyright 1974. All rights reserved

MODIFICATION OF BRAIN SEROTONIN BY THE DIET

❖7089

John D. Fernstrom, Ph.D.
Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts

I. INTRODUCTION

Serotonin is found in a variety of mammalian cells, including the enterochromaffin cells of the gut, pineal parenchymal cells, platelets, and certain brain neurons (1). Within brain neurons, serotonin appears to function as a neurotransmitter (2). The studies to be reviewed show that brain serotonin concentrations change rapidly following the consumption of a single meal. The mechanism of these diet-induced alterations in brain serotonin involves changes in the availability to the brain of L-tryptophan, the amino acid precursor of serotonin.

Since more than 90% of all the serotonin formed in the body is synthesized in the gut (1), the measurement of serotonin metabolites in the urine or blood cannot be taken as an index of brain serotonin turnover. Similarly, the concentration of 5-hydroxyindoleacetic acid (the principal metabolite of serotonin) in lumbar fluid has recently been shown to be an unreliable indicator of brain serotonin turnover (3). Consequently, it is not currently possible to estimate brain serotonin synthesis accurately in humans. For this reason, this review only considers evidence derived from experimental animals.

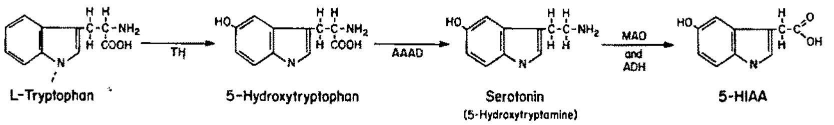


Figure 1 The synthesis and metabolism of serotonin in the brain.

Serotonin Synthesis and Metabolism in the Brain

Serotonin is synthesized from the essential amino acid L-tryptophan (Figure 1). Tryptophan is first hydroxylated to form the amino acid 5-hydroxytryptophan; this compound is then decarboxylated, producing serotonin, or 5-hydroxytryptamine

(5HT). The hydroxylation reaction is catalyzed by an enzyme, tryptophan hydroxylase, found only in serotonin-producing cells (4); the decarboxylation is catalyzed by a widely distributed enzyme that also acts on other substrates such as L-dihydroxyphenylalanine (L-dopa). (5). The principal metabolite of serotonin, 5-hydroxyindoleacetic acid (5HIAA), is formed in a two-step process involving deamination by monoamine oxidase, yielding an aldehyde intermediate, and then oxidation to 5HIAA through the action of aldehyde dehydrogenase (2).

Control of Serotonin Biosynthesis in Brain

Tryptophan hydroxylation appears to be the rate-limiting step in serotonin biosynthesis. The rate at which this process occurs depends not on endproduct inhibition of the hydroxylating enzyme (6) [i.e. such as occurs in the control of catecholamine biosynthesis (7)], but on the availability of its substrate, L-tryptophan. Thus, for example, injections of the amino acid (50–1600 mg/kg) into dogs and rats rapidly elevate blood and brain tryptophan, and brain 5HT and 5HIAA (8).

Such pharmacologic observations by themselves, however, do not constitute adequate proof that brain tryptophan concentrations influence the rate of serotonin synthesis in normal animals. It is also necessary to demonstrate that brain tryptophan levels normally change in untreated animals, and that such changes are capable of affecting 5HT synthesis.

To this end, we attempted to identify spontaneous changes in brain tryptophan in rats by looking for a 24-hr rhythm in brain tryptophan. Daily rhythms had already been described for most amino acids, including tryptophan, in the blood of rats and humans (9), and for brain serotonin in rats (10). This rhythm was found to exist (11): Brain tryptophan concentrations peaked around midnight and attained their nadir early in the light period. The amplitude of the daily variation was almost 100% of the concentration at the nadir.

Since we now knew that brain tryptophan normally varied between 3.5 $\mu g/g$ (morning) and 7 $\mu g/g$ (night), we could determine whether an experimentally induced increase of this magnitude, introduced when brain tryptophan was normally low, could elevate brain serotonin. Groups of rats were injected with very small doses of L-tryptophan (12.5 mg/kg, or less than 5% of the tryptophan that a 150-g rat would normally consume each day in dietary protein) at noon. Within 10 min, both plasma and brain tryptophan attained maximal concentrations, which were maintained for at least 1 hr (12). Neither exceeded the highest normal daily concentrations observed in the rhythm studies. Following this treatment, brain serotonin concentrations increased 20–30% within 1 hr. (The daily rhythm in brain serotonin is about 30% of nadir concentrations.)

These studies thus supported the hypothesis that brain serotonin synthesis normally responds to changes in brain tryptophan levels. Inasmuch as the brain cannot make tryptophan, and must ultimately depend on the plasma for the amino acid, it seemed likely that any factors that altered plasma amino acid concentrations might, by changing the availability of tryptophan to the brain, thereby influence brain serotonin synthesis. We first tested this hypothesis with insulin, because it had been well established that this hormone affects plasma amino acid concentrations.

II. INSULIN AND CARBOHYDRATE

Insulin is secreted in response to the ingestion of protein, carbohydrates, and possibly fats (13, 14). The hormone stimulates the uptake of most amino acids into muscle (15), causing their concentrations in blood to fall. We anticipated that insulin would lower plasma tryptophan, and consequently, brain tryptophan and 5HT in rats. Animals were fasted overnight and injected with insulin (2 U/kg, intraperitoneally) the next day. To our surprise, plasma tryptophan concentrations increased by 40–50% over control values within 2 hr (16). Concurrently, the plasma concentrations of glucose and of most other amino acids declined, as expected. Although this result was unexpected, it nonetheless allowed us to test further the hypothesis that the availability of circulating tryptophan to the brain controls brain tryptophan and 5HT concentrations. In these animals, insulin was found to elicit a 50% rise in brain tryptophan and a 25% increase in brain serotonin (17).

It seemed possible that the elevation of brain serotonin by insulin resulted not from the increase in brain tryptophan, but from some other effect related to the depression in blood sugar. To study this possibility, and to determine whether the rat could manipulate its own brain 5HT level by eating foods that cause insulin to be secreted, we fasted groups of rats overnight, and then gave them free access to a carbohydrate diet for 1–2 hr before sacrifice. The effects of this treatment on tryptophan and serotonin metabolism paralleled those following insulin administration: Plasma tryptophan rose by 40% after 2 hr, brain tryptophan increased by 65%, and brain serotonin rose by 20% (17) (Table 1). Somewhat surprisingly, this study thus indicated that the animal, by choosing to eat a grossly physiological food source, could electively control the concentration of a neurotransmitter in its brain.

Table 1 Effect of dietary carbohydrate ingestion on brain serotonin concentrations and on plasma and brain tryptophan[a]

	Time after food presentation (hours)		
	0	1	2
Plasma tryptophan (μg/ml)	10.86 ± 0.55	13.56 ± 0.81[b]	14.51 ± 0.70[d]
Brain tryptophan (μg/g)	6.78 ± 0.40	8.32 ± 0.63[c]	11.24 ± 0.52[d]
Brain serotonin (μg/g)	0.549 ± 0.015	0.652 ± 0.046	0.652 ± 0.012[d]

[a] Reproduced from (17).
[b] $P < 0.02$ compared with 0-hour control.
[c] $P < 0.05$ compared with 0-hour control.
[d] $P < 0.001$ compared with 0-hour control.

Based on the above findings, we conjectured that the consumption of almost any food should raise brain tryptophan and 5HT, since most foods contain at least some protein or carbohydrate (17). Carbohydrate ingestion (and insulin secretion) would elevate brain serotonin by raising plasma and brain tryptophan, as above. Protein ingestion would presumably increase brain tryptophan and 5HT even further, since plasma tryptophan would be elevated not only in response to insulin secretion, but also to the influx of "new" tryptophan molecules from the diet.

III. PROTEIN

To test the effects of protein ingestion on brain tryptophan and 5HT, we fasted groups of rats overnight, and the next day gave them free access to a carbohydrate diet containing protein (casein, 18% dry weight). Following the consumption of this diet, plasma tryptophan rose even higher than the levels achieved after carbohydrate ingestion. However, *no* increases occurred in either brain tryptophan or 5HT (Figure 2) (18).

These paradoxical results suggested that factors other than the plasma tryptophan concentration must be important in determining tryptophan uptake by brain. One possible factor was competition between tryptophan and other amino acids for uptake sites. Lajtha (19) had shown that amino acids compete for brain uptake, but that the competition is nonuniform: Acidic amino acids (aspartate, glutamate) compete with each other, but not with basic (lysine, histidine) or neutral (trypto-

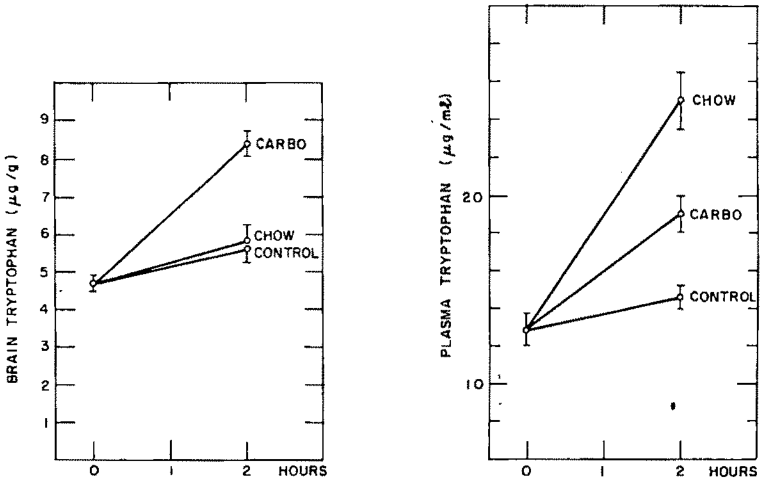


Figure 2 Changes in plasma and brain tryptophan following carbohydrate or protein ingestion. Groups of six rats were killed two hours after diet presentation. Vertical bars represent standard errors of the mean. Two-hour plasma tryptophan is significantly greater in rats consuming either diet than in fasting controls (chow, $P < 0.001$; carbohydrate, $P < 0.01$). Two-hour brain tryptophan is significantly elevated over control only in rats consuming carbohydrate ($P < 0.001$). Reproduced from (18).

phan, tyrosine, phenylalanine, leucine, isoleucine, valine) amino acids. Similarly, neutrals compete with neutrals, but not with acidic or basic amino acids. Thus, we hypothesized that brain tryptophan did not rise in animals consuming protein because the plasma concentrations of competing neutral amino acids were increased even more by the protein than the plasma tryptophan level. This interpretation was compatible with the known scarcity of tryptophan in most proteins: Tryptophan tends to be the least abundant amino acid in dietary proteins (11). According to this formulation, the increase in brain tryptophan following carbohydrate ingestion reflects not only the rise in plasma tryptophan, but also the insulin-induced fall in the plasma levels of the competing neutral amino acids.

To test this hypothesis, we fed rats one of three diets (20): The first contained an amino acid mixture in proportions such that it mimicked an 18% casein diet in amino acid composition; the second differed from the first only in that the competing neutral amino acids (tyrosine, phenylalanine, leucine, isoleucine, and valine) were deleted; the third was missing the acidic amino acids. Consumption of the first or third diets produced results identical to those observed in rats eating an 18% casein diet: Plasma tryptophan rose, but brain tryptophan did not. However, ingestion of the second diet, lacking the competing amino acids, caused large elevations in brain tryptophan, 5HT, and 5HIAA (20).

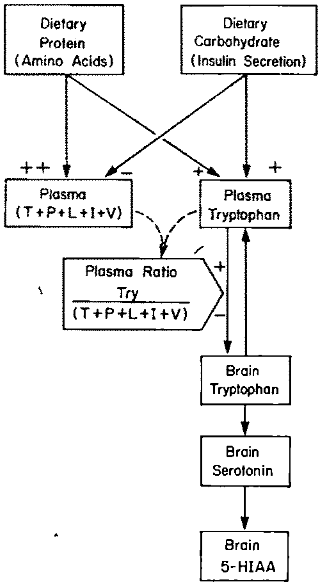

Dietary Protein (Amino Acids)
Dietary Carbohydrate (Insulin Secretion)
++
−
+
+
Plasma (T+P+L+I+V)
Plasma Tryptophan
Plasma Ratio Try / (T+P+L+I+V)
+
−
Brain Tryptophan
Brain Serotonin
Brain 5-HIAA

Figure 3 Proposed sequence describing diet-induced changes in brain serotonin concentration in the rat. The ratio of tryptophan to tyrosine (T) plus phenylalanine (P) plus leucine (L) plus isoleucine (I) plus valine (V) in the plasma is thought to control the tryptophan concentration in the brain. Reproduced from (20).

The dietary control of brain serotonin can be modeled as in Figure 3 (20). Carbohydrate elevates brain tryptophan by simultaneously raising plasma tryptophan and lowering the plasma concentrations of the competing neutral amino acids. If this information is expressed as a plasma ratio of tryptophan to the competing amino acids, carbohydrate elevates brain tryptophan by raising this ratio. Protein consumption increases both plasma tryptophan and the plasma levels of competing amino acids, so that the ratio does not rise, and brain tryptophan and 5HT remain unaffected.

IV. FAT

Tryptophan in blood is distributed between two pools: About 10–20% is present as free amino acid, while the rest circulates bound to serum albumin (21). None of the other amino acids associates with albumin to a significant extent. Because binding often implies storage, a number of investigators have suggested that only the "free" (non-albumin-bound) tryptophan pool determines the availability of circulating tryptophan to the brain (22).

To test this hypothesis, we measured changes in serum free and total tryptophan in rats receiving insulin or consuming carbohydrate, and correlated them with the concomitant alterations in brain tryptophan. In both cases, serum *total* tryptophan and *brain* tryptophan rose markedly. However, serum *free* tryptophan levels actually declined (Table 2) (23). Inasmuch as insulin lowers the serum concentrations of nonesterified fatty acids (NEFA), and these compounds are known to compete with tryptophan for binding to albumin (24), it appears that the decline in serum free tryptophan and the increase in albumin-bound tryptophan that follow these treatments are mediated by insulin effects on serum NEFA concentrations (25).

In other experiments, Madras (26) exploited the competition between NEFA and tryptophan for albumin binding to study further the possible relationship between serum free tryptophan and brain indoles over a wide range of tryptophan concentrations. Groups of rats were fasted overnight, and then given free access to diets containing carbohydrate, protein, and differing amounts of fat. In confirmation of earlier studies on protein-containing diets (section III) (18), none of these diets

Table 2 Effect of dietary carbohydrate ingestion on serum free and bound tryptophan and brain tryptophan concentrations[a]

	Serum tryptophan		Brain tryptophan
	Free (μg/ml)	Total (μg/ml)	(μg/g)
Fasted control	6.2	16.5	2.24
Carbohydrate	3.4[b]	19.1	3.45[b]

[a] Rats deprived of food at 1400 hr were given free access to the experimental diet at 1030 hr the next day and killed 2 hr later. Reproduced from (23).

[b] $P < 0.001$ compared to fasted control.

elicited a rise in brain tryptophan, even though serum NEFA levels and serum free tryptophan concentrations increased in proportion to the fat content of the diet (26). Thus, even though drug-induced changes in the binding of serum tryptophan to albumin may be predictive of alterations in brain tryptophan and 5HT concentrations (22), no such relationship seems to exist under physiologic conditions.

V. CONCLUSIONS

These studies show that diet-induced changes in the plasma concentrations of tryptophan and of other, competing neutral amino acids influence brain tryptophan and 5HT levels. Because of technical limitations, it cannot now be determined whether such alterations in brain serotonin *level* cause corresponding changes in the amounts of the amine *released* into synapses; i.e. when serotonergic neurons are depolarized. It does seem possible to approach this problem indirectly, by asking whether physiologic and behavioral brain outputs thought to be controlled in part by serotonin neurons [e.g. thermoregulation (27), eating behavior (28)] are affected by the consumption of foods that raise brain serotonin.

In human subjects, plasma tryptophan neither increases nor decreases significantly following insulin injection (25). However, the plasma concentrations of competing neutral amino acids do fall markedly (9); hence, the ratio of tryptophan to the competing neutral amino acids increases in humans after insulin, just as it does in rats. Thus, it might be expected that similar mechanisms mediate the dietary control of brain serotonin in rats and man.

Finally, the relationship between brain serotonin and the diet may be clinically important. By feeding patients foods that elevate brain serotonin, it may be possible to affect brain outputs associated with serotonergic neurons (e.g. sleep). It may also be possible to enhance the efficacy of drugs (like the imipramines) that increase the interaction between serotonin molecules and their receptors. Conversely, drugs that block serotonin receptors (like methysergide) may be used to greater effectiveness if patients are concurrently restricted to diets that do not stimulate serotonin synthesis.

ACKNOWLEDGMENTS

These studies were supported in part by grants from the John A. Hartford Foundation and the National Aeronautics and Space Administration.

Literature Cited

1. Garattini, S., Valzelli, L. 1965. *Serotonin.* Amsterdam: Elsevier. 392 pp.
2. Cooper, J. R., Bloom, F. E., Roth, R. H. 1970. *The Biochemical Basis of Neuropharmacology.* New York: Oxford. 220 pp.
3. Bulat, M., Zivkovic, B. 1971. *Science* 173:738–40
4. Lovenberg, W., Jequier, E., Sjoerdsma, A. 1968. *Advan. Pharmacol. A* 6:21–36
5. Lovenberg, W., Weissbach, H., Udenfriend, S. 1962. *J. Biol. Chem.* 237:89–92
6. Millard, S. A., Costa, E., Gal, E. M. 1972. *Brain Res.* 40:545–51
7. Spector, S., Gordon, R., Sjoerdsma, A., Udenfriend, S. 1967. *Mol. Pharmacol.* 3:549–55
8. Moir, A. T. B., Eccleston, D. 1968. *J. Neurochem.* 15:1093–1108
9. Wurtman, R. J. 1970. *Mammalian Protein Metab.* 4:445–79
10. Dixit, B. N., Buckley, J. P. 1967. *Life Sci.* 6:755–58
11. Wurtman, R. J., Fernstrom, J. D. 1972. *Perspectives in Neuropharmacology,* ed. S. H. Snyder, 143–93. New York: Oxford. 404 pp.
12. Fernstrom, J. D., Wurtman, R. J. 1971. *Science* 173:149–52
13. Dupré, J. 1970. *Ann. Rev. Med.* 21:299–316
14. Crespin, S. R., Greenough, W. B., Steinberg, D. 1969. *J. Clin. Invest.* 48: 1934–43
15. Wool, I. G. 1965. *Fed. Proc.* 24:1060–70
16. Fernstrom, J. D., Wurtman, R. J. 1972. *Metabolism* 21:337–42
17. Fernstrom, J. D., Wurtman, R. J. 1971. *Science* 174:1023–25
18. Fernstrom, J. D., Larin, F., Wurtman, R. J. 1973. *Life Sci.* 13:517–24
19. Blasberg, R., Lajtha, A. 1965. *Arch. Biochem. Biophys.* 112:361–77
20. Fernstrom, J. D., Wurtman, R. J. 1972. *Science* 178:414–16
21. McMenamy, R. H., Lund, C. C., Oncley, J. L. 1957. *J. Clin. Invest.* 36:1672–79
22. Knott, P. J., Curzon, G. 1972. *Nature* 239:452–53
23. Madras, B. K., Cohen, E., Fernstrom, J. D., Munro, H. N., Wurtman, R. J. 1973. *Nature* 244:34–35
24. McMenamy, R. H., Oncley, J. L. 1958. *J. Biol. Chem.* 233:1436–47
25. Lipsett, D., Madras, B. K., Wurtman, R. J., Munro, H. N. 1973. *Life Sci.* 12(II):57–64
26. Madras, B. K. In press
27. Myers, R. D. 1968. *Advan. Pharmacol. A* 6:318–21
28. Funderburk, W. H., Hazelwood, J. C., Ruckart, R. T., Ward, J. W. 1971. *J. Pharm. Pharmacol.* 23:468–69

Copyright 1974. All rights reserved

VENEZUELAN EQUINE ENCEPHALITIS VIRUS INFECTION IN MAN

◆7090

N. Joel Ehrenkranz, M.D. and Arnoldo K. Ventura, Ph.D.
Department of Medicine, University of Miami School of Medicine and Cedars of Lebanon Hospital, Miami, Florida

The Venezuelan equine encephalitis (VEE) virus complex consists of closely related virus types found in more or less contiguous areas of South America, Central America, and Southern United States. Disease caused by these types of mosquito-borne group A arbovirus varies considerably in different animal species. Some infect horses and other equines as well as smaller mammals, and cause devastating country wide epizootics of encephalitis; others involve rodents in a limited locale and do not cause disease in equines. Human disease varies greatly in clinical expression.

ANTIGENIC RELATIONS OF VEE VIRUS SUBTYPES

VEE virions are made up of a ribonucleic acid core covered by a protein envelope, and contain larger amounts of phospholipids than the infected host cell (1). Virus infectivity is decreased by treatment with various lipid solvents including sodium desoxycholate, diethyl ether, and various alcohols. These solvents, however, do not release infectious ribonucleic acid from virions, but may affect lipids or lipoproteins on surface structures that are important in attachment of virions to susceptible cells (2, 3). The lipid moiety located near the virion surface may play a role in the adjuvant effect on immune responses by VEE virus infection, as discussed below.

Painstakingly precise studies by Young & Johnson, employing a kinetic hemagglutination inhibition test, have served to delineate certain subtypes within the complex (4). This differentiation is based on more rapid reaction of antibody with homologous antigen than heterologous, and has fundamental epidemiological implications. For example, virus strains isolated at different times in Brazil, Colombia, Panama, Trinidad, Venezuela, and United States have been segregated into different subtypes or groups. By contrast, older isolates from Argentina and Peru, and more recent ones from the Central American epidemic, appear to be identical (5). Three subtypes, IA, IB, and IC, have been found in epidemics or epizootics, whereas the other subtypes, ID, IE, and groups II, III, and IV, have been associated only with endemic or enzootic infection.

ANIMAL INFECTION WITH DIFFERENT VEE VIRUS GROUPS

High level viremia ($10^6 - 10^8$ plaque forming units per ml) in equines, resulting in heavy infestation of mosquitoes of many species, is the major if not sole determinant of epidemic potential (6). Although VEE virus may be found in the pharynx, person-to-person transmission does not seem to play a role in epidemic spread (7). High level viremia, however, is not the determinant of pathologic changes. For example, in experimental infection of hamsters, both virulent and avirulent strains produced similar high levels of virus in blood, as well as in brain, bone marrow, spleen, and lymph nodes, with the virulent strain attaining high titer somewhat more rapidly (8). Infection with virulent strain resulted in massive necrosis in lymph nodes, intestinal lymphatic tissue, splenic white pulp, and bone marrow, with almost complete destruction of parenchyma of bone marrow, lymph nodes, and spleen by day 4. The attenuated strain, however, caused only minor depletion of lymphocytes in lymph nodes and spleen, but did induce proliferation of splenic reticuloendothelial cells. Further, a horse experimentally infected with the Florida (II) group had histologic findings of encephalitis, although this virus causes only low level viremia in equines and no fatalities (9).

Host differences are clearly important. Guinea pigs and rabbits infected by a number of routes all died in 2 to 4 days. A major pathological finding was extensive necrosis of lymphoid tissues. Monkeys similarly infected did not die, but when sacrificed between 8 and 51 days had histologic findings of encephalitis, characterized by perivascular round cell infiltration, swelling of capillaries, edema, and degeneration of ganglion cells (10, 11). Thus, infection of less susceptible hosts, or with less virulent virus, as reflected by survival, may result in encephalitis. This suggests host responses play an important role in pathogenesis of encephalitis. Pertinent to this is the observation of Berge et al that passive immunization of hyperimmune serum in experimentally infected monkeys, administered when virus titers were at a peak in brain, did not prevent infection but hastened the onset of encephalitis (12).

An adjuvant effect of VEE virus infection on immune response has been demonstrated. This was indicated by increased clearance of foreign antigens and enhancement of antibody titers to them (13). Lymphocyte proliferation in the reticuloendothelial system as a result of this infection may play a role in the adjuvant effect. After avirulent strain infection of guinea pigs, VEE virus antigens were found in liver, spleen, lymph nodes, and kidney at 1–2 days and again at 7–15 days. The second peak was often marked by an almost confluent organ involvement in the reticuloendothelial system, with increase in follicular lymphocytes and enlargement of germinal centers (14).

VEE MANIFESTATIONS IN MAN

While a great deal of human infection may not be clinically apparent, recognized disease due to VEE virus infection can be categorized into three forms: 1. *influenzal* —manifested by constitutional symptoms alone, except for pharyngitis, with a febrile course of 1 to 4 days. Virus present in blood and pharynx (15) is likely to be responsible for some signs and symptoms: neither lymphocytolytic effects nor autoimmune phenomena are prominent in symptomatology, although lymphocytopenia may occur. 2. *fulminant*—characterized by a short febrile course, a few days or less, with rapid progression to shock, coma, and convulsions. A radiation-like destruction of lymphocytic tissue is present at autopsy. Survivors may have sequelae, probably as a result of thrombosis secondary to shock. This type of disease appears to be due to lymphocytolytic effects of highly virulent strains of VEE virus. Virus is present in blood and organs. 3. *encephalitic*—evidenced by a febrile course often 2 weeks or more, sometimes diphasic. Central nervous system manifestations begin in the second phase, after several days to a week of prodromal illness. The encephalitic form may be due to autoimmune responses of the host. Viremia and nonfatal lymphocytopenic effects are probably present early. Recovery is usually complete.

Bearing these three clinical presentations in mind, we will analyze various outbreaks and episodes of VEE virus infection. All clinical information about VEE infection in man prior to 1952 derived from accidental infections with epizootic subtypes.[1] In these persons, who were generally young adults, clinical manifestations were influenzal: abrupt onset of illness with chills, headache, myalgia, lethargy, prostration, vomiting, diarrhea, somnolence, and often pharyngitis. Temperature elevations were 101 to 104°F. No major neurological changes were observed (17–19).

Fully documented natural epidemic infection of man was first described in 1952 in Colombia (20). The disease was influenzal in adults, but one 8-year-old child did have neurologic manifestations: convulsion, loss of memory, and changes in behavior. Subsequently, serious disease was also noted in children in other epidemics. The epidemic in Zulia, Venezuela in 1962 had a clinical attack rate of 2:1 in persons under 15 years as compared with those 15 and older; in 44 deaths the ratio was 37:7 in these age groups (21). By 1964 when the epidemic ran its course in the whole country it is estimated there were 32,000 cases and 190 deaths—the latter mostly in children (22). In Mexico in 1971 there were some 16,900 cases in persons of all age groups, the greater severity again in children. Age distribution of 42 fatal cases was: 75% in children under 5 years, 20% in those 5 to 14 years, and 5% in those over 45 years. No deaths were reported in persons age 15 to 44 (23). Since disease rates were higher in children than in adults, it was suggested that adults had immunity as a result of prior exposure. Although some VEE virus infection of man had occurred with an enzootic strain in Mexico in 1966 (24), epidemic disease was

[1] Reports of human disease in 1944 are not now accepted as VEE (16).

not recorded. Hence, it is unlikely that the predominance of serious infection in children in Mexico in 1971 was due to widespread adult immunity.

In El Salvador and Guatamala in 1969, the most serious clinical disease was also reported in children, as reflected by disorientation, stupor, and occasional death. A serological survey indicated widespread VEE virus infection, but no excess of infected children to indicate adult immunity (25).

In the United States in 1971, the epidemic strain causing human disease in Texas generally resulted in influenzal manifestations in adults. Preliminary reports, however, reveal that some children were severely affected (26). A 6-year-old girl was comatose for 1 week and had multiple seizures. Early sequelae included behavioral changes, partial paralysis of a leg, and bilateral 6th nerve palsies. A 5-year-old girl was left with twitching of muscles and distortion of the left side of her mouth. The severity of disease in children was not due to preexisting adult immunity, since this clearly was the first epidemic incursion of VEE virus into Texas.

Thus, in epidemic VEE virus infection, fulminant disease leading to death or sequelae is prominent in children. We suggest that persons under age 15, like guinea pigs, are especially susceptible to the lymphcytotoxic effects of VEE virus infection. Persons 15 to 45 years are less susceptible to lymphocytotoxicity and experience influenzal manifestations of viremia and virus proliferating in the pharynx. Older individuals may experience encephalitis like monkeys, or serious disease due to preexisting illness.

In contrast to the large numbers of people involved in epidemic VEE, only 14 cases of endemic VEE have been recognized thus far. Of these, 8 cases occurred in Panama, 3 in Mexico, and 3 in the United States. Although none were in very young children, 2 persons died, and 4 had signs of encephalitis. In Panama, 7 U.S. Army personnel became ill during field manuvers (27). They all experienced influenzal symptoms and viremia. Virus was present in throat swabs of 2. All 7 persons manifested leucopenia with marked lymphocytopenia, only one had a sign of encephalitis—transient delirium. By contrast, endemic VEE was fatal to a 14-year-old boy in Panama, his 3-day illness terminating in convulsions, coma, and irreversible shock (28). At autopsy, findings of cerebral edema with occasional perivascular cuffing were noted in the brain, along with slight interstitial myocarditis. There was marked depletion of lymphoid elements in the spleen and lymph nodes. In Mexico, a probable case of endemic VEE infection in a 28-year-old man was characterized by initial high fever (40°C) and a prolonged diphasic course with stupor ending in coma and death (29). Two other cases in Mexico, in persons age 21 and 40 years, were manifested only by influenzal symptoms and viremia, and pharyngeal virus in one (30).

The three cases of endemic VEE disease in the United States occurred in Florida, and were studied in some detail. The first case was in a 53-year-old woman with preexisting hypertension (31). She had a 2-week illness with malaise, fatigue, anorexia, headache, feverishness, and shaking chills without lymphadenopathy or pharyngitis, which was followed by headache and stupor, progressing to semicoma. No lymphocytopenia was present: initial white blood cell count was 11,300/mm^3 with

28% lymphocytes. Cerebrospinal fluid pleocytosis was 900 white cells/mm^3 with 65% mononuclear cells. EEG changes were those of diffuse encephalitis. She made a full recovery.

A second probable case was in a 71-year-old winter visitor to Vero Beach in 1968 (30); he had preexisting cerebrovascular disease. After onset of disease his mental confusion rapidly progressed to coma. No lymphadenopathy or pharyngitis was noted, although conjunctivitis was present. Lymphocytopenia was present: white blood cell count was 8700/mm^3 with 15% lymphocytes. Cerebrospinal fluid revealed 20 lymphocytes/mm^3. He recovered with no new residua.

The third case in 1971 was in a 75-year-old man in South Miami who had minimal preexisting generalized arteriosclerosis (32). His illness was characterized by a prodrome of malaise, myalgia, nuchal rigidity, fever, and cough without pharyngitis, followed by lethargy and semicoma. EEG abnormalities were those of diffuse cerebral dysrhythmias. Lumbar puncture revealed only 12 white cells/mm^3. No pharyngitis was present. Lymphocytopenia was noted—initial white blood cell count was 6700/mm^3 with 2% lymphocytes. Recovery was complete.

These three middle-aged or elderly individuals had VEE virus infection characterized by clinical and laboratory evidence of cerebral involvement. All had some measure of generalized preexisting vascular disease. None had pharyngitis or lymphadenopathy. Lymphocytopenia was prominent in two. One patient had a prodromal illness of 2 weeks before encephalitis became apparent. The place of acquisition of infection by these three individuals was not established, but two lived close to the coast and had no exposure to known inland focal sites of enzootic VEE virus activity. Whether VEE virus in enzootic inland focal sites in Florida is less virulent for man than isolates infecting people elsewhere, or involves a less susceptible population, is not known at present. Zarate & Scherer (33) observed differences in virulence in experimental studies employing different enzootic VEE virus isolates from Mexico.

Thus, in the handful of endemic VEE cases recorded, a pattern of disease manifestations similar to those of epidemic VEE was observed. Fulminant disease was found in an individual under 15 years old. Classic encephalitis occurred in 3 persons over age 50. Influenzal disease was predominant in persons between 15 and 50 years, although 1 person in this age group did have diphasic illness with a fatal outcome and another had delirium. The absence of serious disease in young children is due to the absence of endemic VEE virus infection in them. Serological studies in Florida[2] and Mexico reveal very little infection in persons under 10 years old (34).

In summary, we feel that the lymphocyte is central to the pathogenesis of human disease after VEE virus infection. Profound destruction of lymphocytes in individuals under age 15 may result in fulminant disease whether caused by virulent endemic or epidemic virus types. Less profound lymphocytopenia, followed by lymphocyte and reticuloendothelial cell proliferation, leads to a diphasic illness in persons over age 50 which may be manifested by encephalitis. It is likely that an autoimmune

[2] Ventura, A. K., Buff, E., Ehrenkranz, N. J. Unpublished observations.

mechanism is involved, and in this the adjuvant effect of VEE virus infection on immune responses could play a role. In the main, however, VEE virus infection in persons between 15 and 50 years is benign, influenzal if symptomatic at all, with virus in blood and pharynx accounting for most of the signs and symptoms of the acute illness.

Literature Cited

1. Heydrick, F. P., Comer, J. F., Wachter, R. F. 1971. *J. Virol.* 7:642–45
2. Ventura, A. K., Scherer, W. F. 1970. *Proc. Soc. Exp. Biol. Med.* 133:711–17
3. Ventura, A. K., Scherer, W. F. 1970. *Proc. Soc. Exp. Biol. Med.* 134:583–90
4. Young, N. A., Johnson, K. M. 1969. *Am. J. Epidemiol.* 89:286–307
5. Young, N. A. 1972. *Venezuelan Encephalitis.* Sci. Publ. No. 243, PAHO, Washington, D.C., p. 84–89
6. Sudia, W. D., Newhouse, V. F., Henderson, B. E. 1971. *Am. J. Epidemiol.* 93:206–11
7. Sanmartin, C. 1972. *Venezuelan Encephalitis.* Sci. Publ. No. 243, PAHO, Washington, D.C., p. 186–88
8. Austin, F. J., Scherer, W. F. 1971. *Am. J. Pathol.* 62:195–210
9. Henderson, B. E., Chappell, W. A., Johnston, J. G., Sudia, W. D. 1971. *Am. J. Epidemiol.* 93:194–205
10. Victor, J., Smith, D. G., Pollack, A. D. 1956. *J. Infec. Dis.* 98:55–56
11. Gleiser, C. A., Gochenour, W. S., Berge, T. O., Tigertt, W. D. 1962. *J. Infec. Dis.* 110:80–97
12. Berge, T. O., Gleiser, C. A., Gochenour, W. S., Miesse, M. L., Tigertt, W. D. 1961. *Am. J. Hyg.* 87:509–17
13. Craig, C. P., Reynolds, S. L., Airhart, J. W., Staab, E. V. 1969. *J. Immunol.* 102:1220–27
14. Airhart, J. W., Trevino, G. S., Craig, C. P. 1969. *J. Immunol.* 102:1228–34
15. Briceño Rossi, A. L. 1964. *Rev. Venez. Sanid. Asistencia Soc.* XXIX:393–409
16. Tigertt, W. D., Downs, W. G. 1962. *Am. J. Trop. Med. Hyg.* 11:822–34
17. Casals, J., Curnen, E. C., Thomas, L. 1943. *J. Exp. Med.* 77:521–30
18. Lennette, E. H., Koprowski, H. 1943. *J. Am. Med. Assoc.* 123:1088–95
19. Sutton, L. S., Brooke, C. C. 1954. *J. Am. Med. Assoc.* 155:1473–76
20. Sanmartin-Barberi, C., Groot, H., Osorno-Mesa, E. 1954. *Am. J. Trop. Med. Hyg.* 3:283–93
21. Castillo, C. E. 1964. *Rev. Venez. Sanid. Asistencia Soc.* XXIX:325–48
22. Sellers, R. F., Bergold, G. H., Suarez, O. M., Morales, A. 1965. *Am. J. Trop. Med. Hyg.* 14:460–69
23. Avilan Rovira, J. 1972. *Venezuelan Encephalitis.* Sci. Publ. No. 243, PAHO, Washington, D.C., p. 189–92
24. Morilla-Gonzales, M. V. Z. A., Mucha-Macias, J. 1969. *Rev. Invest. Salud Publica* 29:3–20
25. Hinman, A. R., McGowan, J. E., Henderson, B. E. 1971. *Am. J. Epidemiol.* 93:130–36
26. Bowen, G. S. 1972. *Venezuelan Encephalitis.* Sci. Publ. No. 243, PAHO, Washington, D.C., p. 231–34
27. Franck, P. T., Johnson, K. M. 1970. *Am. J. Trop. Med. Hyg.* 19:860–65
28. Johnson, K. M., Shelokov, A., Peralta, P. H., Dammin, G. J., Young, N. A. 1968. *Am. J. Trop. Med. Hyg.* 17:432–40
29. Zarate, M. L., Scherer, W. F., Dickerman, R. W. 1971. *Salud Publica Mex.* 13:97–99
30. Scherer, W. F. et al 1972. *Am. J. Trop. Med. Hyg.* 21:79–85
31. Ehrenkranz, N. J., Sinclair, M. C., Buff, E., Lyman, D. O. 1970. *N. Engl. J. Med.* 282:298–302
32. Ventura, A. K., Ehrenkranz, N. J., Saslaw, M. S., Buff, E. E., Schneider, N. J. 1971. *Morbid. Mortal. Weekly Rep.* 20:411–12
33. Zarate, M. L., Scherer, W. F. 1969. *Am. J. Epidemiol.* 89:489–502
34. Mucha-Macias, J., Sanchez-Spindola, I., Campillo-Sainz, C. 1966. *Am. J. Trop. Med. Hyg.* 15:364–68

Copyright 1974. All rights reserved

GENETIC ASPECTS OF GOUT ❖7091

Michael A. Becker, M.D. and J. Edwin Seegmiller, M.D.[1]
Department of Medicine, University of California - San Diego, La Jolla, California

INTRODUCTION

At the present time medicine faces a curious dichotomy in the depth of its understanding of hereditary diseases that seems out of harmony with the ground swell of desire for more effective delivery of health care. An increasing number of the rare and exotic recessively inherited diseases are rapidly being characterized as deficiencies of specific enzymes and provide valuable models of gene action. Yet, the specific hereditary factors underlying the more common, usually dominantly inherited disorders such as diabetes, hypertension, cardiovascular disease, mental illness, and some forms of arthritis continue to elude intensive investigations. In this presentation we will delineate the beginning that has been made in the identification of specific genetically determined enzyme abnormalities and biochemical mechanisms responsible for the development of gouty arthritis. Since one of these well-defined genetic defects apparently is dominantly inherited, it may well set a pattern for an approach to other more common dominantly inherited diseases.

As a disorder well known in antiquity, gout was recognized quite early as a familial disease (1). Only when the biochemical basis of the malady was identified, however, was an ordered approach to the genetics of gout possible. Thus, the identification of uric acid renal stones by Scheele (1776) and the classical demonstration of hyperuricemia in gouty individuals by A. B. Garrod in 1848 (2) provided the impetus to the study of purine metabolism, which eventuated in the concept of gout as a clinical expression of the excessive accumulation of a normal but relatively insoluble metabolic product, uric acid.

In 1931, A. E. Garrod included gout among the inborn errors of metabolism (3), a class of hereditary metabolic abnormalities which he had first proposed in the Croonian Lectures of 1908 (4). From his clinical observations, Garrod concluded that gout was a dominantly inherited trait, incompletely expressed especially in women (3). Moreover, he suggested that an even greater number of hyperuricemic but asymptomatic persons would be found among the relatives of gouty individuals.

---

[1] The authors acknowledge support from grants AM 13622, AM 05646, and GM 17702 from the National Institutes of Health and by grants from the National Genetics Foundation, the San Diego County Heart Association, and the National Foundation.

While this latter prediction has been amply confirmed (5), the dominant trait hypothesis incorrectly assumed a unitary genetic aberration leading to hyperuricemia and gout. Several studies of the families of gouty patients were carried out on this assumption and appeared initially to provide evidence for autosomal dominant inheritance of hyperuricemia and gout with variable penetrance between sexes (6, 7). Later studies, however, contradicted these findings and indicated polygenic control of serum urate concentration (8, 9). The contradictory results and interpretations may have reflected the experimental designs, with longitudinal studies of the concordance of hyperuricemia in successive generations revealing those families with an autosomal dominant transmission and studies conducted among siblings and cousins of affected persons emphasizing other patterns of inheritance. Hauge & Harvald (8) anticipated future developments, writing in 1955:

> Continued studies on the serum urate levels in members of families with hyperuricemia can hardly contribute more towards elucidating the problems of the heredity of this condition. More would probably be gained by studying the individual steps of uric acid synthesis as well as its excretion in the individuals of affected families. This might lead to an isolation of etiologically well-defined disease units, the heredity of which could then be determined.

Fortunately, this recognition coincided with a period of rapid development in our knowledge of purine metabolism and in the application of isotopic techniques for determining uric acid pool size and dynamics (10) and the incorporation of labeled precursors into uric acid in normal and gouty subjects (11). Patients with gout showed a variety of mechanisms responsible for the development of hyperuricemia (12–17). Demonstration of a subgroup of gouty individuals with excessive uric acid synthesis suggested aberrations in control of the rate of purine synthesis in these individuals. In the past several years, four specific enzyme defects responsible for excessive purine production and the development of gouty arthritis have been identified (18–22). In a similar fashion, recognition that many gouty individuals do not synthesize excessive quantities of uric acid has focused attention on a loose binding of urate to plasma proteins (23, 24) and on defects in renal excretion of uric acid. A deficiency of a genetically determined $\alpha 1$-$\alpha 2$ globulin of plasma has been found in patients who developed gout in two hyperuricemic families (23). Primary genetic defects in either carbohydrate (18) or amino acid (25) metabolism have also been found in which hyperuricemia resulted from an inhibition of renal excretion of uric acid mediated by accumulation of organic acids (26). In addition, hypouricemia resulting from specific defects in renal tubular reabsorption has been found in asymptomatic individuals (27–29) as well as in patients with such disorders as Wilson's disease (30), cystinosis, and other diseases associated with the renal Fanconi syndrome (31).

Abnormalities in the serum urate concentration thus become relatively nonspecific indicators of a wide variety of primary genetic abnormalities. Detailed study of the mechanisms of purine overproduction in patients with known specific defects of enzymes of purine metabolism has produced a unified concept of the types of aberrations in the normal regulatory mechanism that can lead to excessive purine synthesis. It has also allowed more rational approaches to the study and treatment of this disease state. For example, the clinical identification of patients who produce excessive quantities of uric acid[2] allows their rational treatment with allopurinol, which not only blocks uric acid production by inhibiting xanthine oxidase but in most cases also diminishes the excessive rate of purine synthesis (32).

Major emphasis will be given here to well-defined hereditary aberrations in gout as they are presently understood. The predominance of discussion of the heredity of purine overproduction reflects the contemporary state of knowledge; relatively little is yet known of the genetic determinants of plasma binding (23, 24), tissue deposition (33), or renal excretion of urate (34).

PATHOGENETIC CONSIDERATIONS

Uric acid is the end product of purine metabolism in man and certain other primates who are unusual among mammals in their inability to oxidize uric acid to the more soluble product, allantoin. The result of this species-wide deficiency of the enzyme uricase is the necessity of excreting uric acid, which is relatively insoluble in the physical-chemical milieu of body fluids. The relatively inefficient renal mechanism for excretion of uric acid in the human may be of some value in smoothing out the surges of high concentration of uric acid which would otherwise follow ingestion of high-purine foods, and it thereby diminishes the chances of formation of concretions of uric acid in the urinary tract. Nevertheless, it results in a mean concentration of urate in the serum of normal males of 5.1 mg per 100 ml, which approaches the theoretical limit of solubility in serum of 6.4 mg per 100 ml (35). Hyperuricemic serum is therefore theoretically supersaturated, and the likelihood of precipitation of crystals of monosodium urate, pathognomonic for gout, in and about the joints, tendons, and interstitium of the renal pyramids and subcutaneous tissues, increases markedly as the degree of hyperuricemia increases (36). Gout may be considered a clinical syndrome of multiple etiologies with a pathogenetic common denominator, hyperuricemia, which is necessary but not always sufficient for expression of the disease state. Although disruption of lysosomal membranes by crystals of monosodium urate may be the molecular basis of the inflammatory reaction (37, 38), the factors, genetic or otherwise, determining the development of clinical gout in a particular hyperuricemic individual at a particular time remain poorly delineated.

[2] A relatively simple method for this assessment is the measurement (conveniently done during recovery from an acute attack) of the uric acid and creatinine excreted in the 24-hr urine during the last two or three days of a five- or six-day diet essentially free of purines. Protein of the diet is limited to eggs and dairy products with elimination of meat, fish, fowl, peas, beans, beer, and beverages or foods containing methylated purines such as coffee, tea, or chocolate. Agents causing a renal retention of uric acid such as salicylates, most diuretic drugs, and alcohol as well as uricosuric drugs are also eliminated. Excretion of more than 600 mg uric acid per day indicates an excessive rate of purine synthesis (17). Urine is collected and stored at room temperature using 2 ml toluene as a preservative prior to analysis.

HEREDITARY BASES OF EXCESSIVE URIC ACID BIOSYNTHESIS

Purine nucleotide synthesis in man involves the closely related, complex interplay of two alternative pathways. In the pathway of purine biosynthesis de novo, small molecule precursors of uric acid are incorporated into a purine ring, which is synthesized on a ribose-phosphate backbone donated by 5-phosphoribosyl-1-pyrophosphate (PRPP). This pathway includes ten enzymatic reactions and requires 6 moles ATP for each mole of inosinic acid generated. The first reaction committed to purine nucleotide synthesis de novo is catalyzed by PRPP amidotransferase, an enzyme whose allosteric regulatory properties (39) appear to agree well with a rate-determining role for this reaction in the pathway. PRPP amidotransferase activity is inhibited by the purine nucleotide products of the pathway; this feedback inhibition is reversed by PRPP, a substrate usually present in limiting concentrations in the cell (40) and whose substrate kinetics show cooperative binding effects in the presence of nucleotides (39). The recent, elegant studies of Holmes, Wyngaarden & Kelley have delineated a molecular basis for the interaction of inhibitory nucleotides and PRPP on PRPP amidotransferase activity (41). Sucrose density gradient centrifugation studies indicate an active form of the enzyme of ∼ 133,000 daltons and an inactive form of the enzyme of 270,000 daltons. The reversible conversion of the active to the inactive form of PRPP amidotransferase can be accomplished by increasing concentrations of inhibitory purine nucleotides; and the effect can be blocked by increasing concentrations of PRPP. These findings appear to confirm in molecular terms a large body of evidence suggesting roles for the intracellular concentrations of purine nucleotides (42–44) and PRPP (21, 22, 40, 45–54) in the regulation of the rate of purine synthesis de novo.

The second pathway of purine nucleotide biosynthesis involves the two enzymes adenine phosphoribosyltransferase and hypoxanthine guanine phosphoribosyltransferase (APRT and HGPRT), which catalyze the synthesis of purine nucleotides from adenine and hypoxanthine or guanine respectively by reactions with PRPP. The role of PRPP as a common substrate in both pathways makes it likely that the interrelationship between salvage and de novo pathways is governed at least in part by the availability of PRPP as well as by the concentrations of the nucleotide products common to the pathways. A single mole of ATP (utilized in PRPP synthesis) is required for synthesis of 1 mole of purine nucleotide by means of this salvage pathway, a considerable energy savings to the cell when compared with the energy requirements of purine synthesis de novo. The greater affinities for PRPP of the enzymes utilizing this substrate for purine reutilization and the lesser affinity of the amidotransferase enzyme in human cells establishes a basis for priorities with preferential utilization of PRPP for purine base salvage (54).

Alterations in the regulatory mechanisms described above appear to explain the biochemical findings in several distinct enzyme abnormalities associated with purine overproduction and gout. In certain other situations, a relationship between an enzyme abnormality and purine overproduction is plausible but as yet unproved.

Table 1 shows a list of these proposed enzyme abnormalities classified according to the inferred mechanism of resulting uric acid overproduction.

PRPP Availability

In addition to the recent studies of PRPP amidotransferase mentioned previously (39, 41) several clinical studies (55–57) and a wealth of pharmacologic data (40, 45–52) show a dependence of the rate of purine synthesis on the availability of PRPP. The incorporation of ^{14}C-glucose into the ribose moiety of urinary imidazole acetic acid ribonucleoside revealed an increased rate of PRPP turnover in three patients with gout and excessive uric acid excretion as compared to control subjects or patients with gout and normal purine excretion (55). In addition, the intracellular concentration of PRPP in erythrocytes and fibroblasts (58, 59) and the generation of PRPP by erythrocytes was significantly increased in a proportion of patients with gout (56, 57).

A number of pharmacologic agents which utilize or increase generation of PRPP produce corresponding alterations in purine synthetic rates. Administration to volunteers of allopurinol, adenine, or orotic acid, which diminish erythrocyte PRPP concentrations, decreases either the 24-hr excretion of urinary purines or the rate of incorporation of labeled glycine into urinary uric acid (45–49). Similarly, incubation of human fibroblasts in tissue culture with adenine, orotic acid, or nicotinic acid

Table 1 Proposed enzyme abnormalities resulting in excessive uric acid synthesis in man

Mechanism	Enzyme Abnormality	Heredity	References
Increased PRPP production	Increased PRPP synthetase activity		21, 60
	1. Diminished response to feedback inhibition	–	
	2. Increased specific activity of enzyme	Probably autosomal dominant	22, 61
	Glucose 6-phosphatase deficiency (Type-I glycogenosis)	Autosomal recessive	18, 63, 64
	Increased glutathione reductase activity	Autosomal dominant	65–67
Decreased PRPP utilization	Severe HGPRT deficiency (Lesch-Nyhan syndrome)	X-linked recessive	19, 72–80, 86
	Partial HGPRT deficiency	X-linked recessive	20, 70, 88–90
Altered feedback inhibition	PRPP amidotransferase relatively insensitive to feedback inhibitors	Unknown	59
	Adenosine kinase deficiency[a]	–	–

[a] Described in 3T6 mouse fibroblast cells (113); human correlate not yet identified.

produces decreases in both PRPP concentrations and rates of purine synthesis de novo (40, 50, 51); incubation of these cells with methylene blue, which increases the rate of the pentose phosphate shunt, results in both increased PRPP concentration and an increased rate of purine synthesis de novo (52). Study of the known inborn errors of purine metabolism has provided further confirmation of the critical regulatory of PRPP.

Increased PRPP Production

An increased rate of PRPP generation has been proposed as the mechanism for purine overproduction in three different enzyme abnormalities associated with gout (Table 1). However, only in the case of increased PRPP synthetase activity is the evidence conclusive. Nevertheless, any genetic alteration capable of accentuating the rate of ribose 5-phosphate production through the pentose phosphate shunt pathway could potentially result in the increased PRPP generation.

INCREASED PRPP SYNTHETASE ACTIVITY This enzyme catalyzes the synthesis of PRPP from ATP and ribose 5-phosphate in a reaction dependent upon inorganic phosphate and magnesium. Two families in whom gout and excessive uric acid production are associated with different structural alterations in PRPP synthetase have recently been described (21, 22). In both families, increased activities of erythrocyte PRPP synthetase have been accompanied by increased generation of PRPP. These findings were corroborated in cultured fibroblasts from affected members of one family in whom an excessive rate of purine synthesis was demonstrated both in fibroblasts and in vivo (22). In addition, the concentrations of PRPP both in fibroblasts and erythrocytes of these individuals were increased about twofold.

PRPP synthetase from the affected patients described by Sperling, deVries and associates demonstrated increased activity only at phosphate concentrations below 2 m*M* (21). This mutant enzyme was altered in its regulatory properties, making it less sensitive than normal to inhibition of activity by a wide range of phosphorylated compounds including purine nucleotides (60). In contrast, feedback inhibition of enzyme activity was normal in the PRPP synthetase from three affected members of the family described by Becker, Seegmiller and collaborators, and the increased enzyme activity was demonstrable at all phosphate concentrations (22). The mutant enzyme in this family showed increased electrophoretic mobility and, by immunochemical studies, an increased activity per molecule (61). Thus, the molecular basis for increased enzyme activity in each of these families is quite distinct, and each represents a type of enzyme alteration not previously described in man. The association in two families of increased enzyme activity with increased PRPP production and purine overproduction strongly suggests a causal role for the increased PRPP synthetase activity in the development of gout by these patients. A primary increase in enzyme activity as a pathogenetic mechanism of human disease is uniquely exemplified by these families.

Hereditary transmission of the abnormality in PRPP synthetase activity appears to be dominant in at least one of the families described (22) because increased enzyme activity is expressed in a daughter of one affected patient whose wife has normal enzyme activity. Since enzyme activity in erythrocytes from this girl is in the range of her affected father, autosomal transmission of the trait seemed likely. For reasons to be discussed later, however, X-linked inheritance of increased PRPP synthetase activity cannot yet be completely excluded on the basis of enzyme determinations in erythrocytes.

GLUCOSE 6-PHOSPHATE DEFICIENCY . The high incidence of gout among patients with glucose 6-phosphatase deficiency (Type-1 glycogen storage or Von Gierke's) disease surviving to adult life results from hyperuricemia produced by at least two biochemical effects of the enzyme deficiency. Interference with renal secretion of uric acid due to the hyperlactic-acidemia and possibly ketonemia from hypoglycemia accounts in part for the development of hyperuricemia (26, 62). In addition, an increased rate of synthesis of uric acid has also been consistently demonstrated by ^{14}C-glycine incorporation studies (18, 63, 64). Increased PRPP production, resulting from excessive activity of the hepatic pentose phosphate shunt pathway in the face of increased glucose 6-phosphate availability, has been hypothesized as a driving force to increased purine synthesis (63). While erythrocyte PRPP concentrations from affected patients have been normal (58), the limited tissue distribution of glucose 6-phosphatase makes it unlikely that the defect would be demonstrable in red blood cells. Confirmation of the hypothesis awaits studies of PRPP concentration and generation in liver tissue from individuals with this autosomal recessive genetic disorder.

INCREASED GLUTATHIONE REDUCTASE ACTIVITY Although an increased activity of a variant form of glutathione reductase has been described in association with hyperuricemia and gout (65–67), recent observations concerning activation of glutathione reductase by flavin adenine dinucleotide (68) have raised questions concerning the accuracy of all previous measurements of this enzyme. While this enzyme has been hypothesized to increase ribose 5-phosphate (and potentially PRPP) generation by increasing the rate of the pentose phosphate shunt (67), a rate-determining role for this enzyme reaction is unlikely (69). In addition, no characteristics of the patients with gout and the variant enzyme have been published, and association of the variant enzyme with gout has not yet been confirmed by other investigators.

Decreased PRPP Utilization

Since PRPP is a substrate in the phosphoribosyltransferase reactions involved in purine, pyrimidine, and pyridine nucleotide synthesis as well as in the PRPP amidotransferase reaction and in the conversion of imidazole acetic acid to its nucleotide, deficiency of any of the respective enzymes could potentially result in increased availability of PRPP for other reactions. Relative affinities for PRPP, the availability of cosubstrates, and the varying roles of each reaction in determining the rate of its particular pathway must, however, govern the metabolic consequences of such deficiencies. In the case of HGPRT, both severe and partial deficiencies result in increased PRPP concentrations and are accompanied by inversely proportional increases in the rate of purine synthesis de novo in all tissues that have been examined (53, 54, 58, 70). Evidence to date suggests decreased utilization of PRPP rather than increased production of this compound as the factor responsible for increased PRPP concentrations (53) and no evidence has been found for nucleotide deficiency in cells deficient in HGPRT (53).[3] The marked increase in intracellular PRPP concentration produced by HGPRT deficiency as well as the absence of any similar known effect resulting from deficiency of other PRPP utilizing enzymes implies a high rate of normal utilization of PRPP by the HGPRT enzyme—perhaps in the modulation, proposed by Atkinson & Fall (71), of the cellular "energy charge."

SEVERE HGPRT DEFICIENCY (LESCH-NYHAN SYNDROME) Both biochemically and clinically, this disorder is the most striking of the known inborn aberrations of purine metabolism. Affected males manifest some or all of the constellation of choreoathetosis, spasticity, compulsive self-mutilation, and mental retardation in addition to a constant markedly excessive uric acid excretion (72). The 24-hr excretion of uric acid in urine in relation to body weight is four to six times greater than in normal adults, and isotope labeling studies have shown a 20-fold greater incorporation of ^{14}C-glycine into urinary uric acid in these children than among controls (70). The degree of overproduction of uric acid is therefore far greater than has been found in any other group of human subjects. The deficiency of HGPRT activity associated with the Lesch-Nyhan syndrome is severe (73) in all organs but not complete (74), and studies using antibodies to HGPRT have consistently demonstrated a normal amount of cross-reacting material in the cells of affected children (75, 76). Furthermore, evidence of a variety of distinct mutational events giving rise to different structural variants of HGPRT in different families has been found (76, 77). Among these variants, proteins with different amounts of activity, altered electrophoretic mobilities, altered sensitivities to heat inactivation or product inhibition, and even altered substrate affinities have been identified (74, 76, 77). Thus, both in this disorder and in increased PRPP synthetase activity, heterogeneity in mutational events giving rise to similar enzyme abnormalities and phenotypic patterns is demonstrable.

Deficiency of HGPRT is an X-linked trait. This is shown both by pedigree (78) and by demonstration of two populations of cells (HGPRT$^+$ and HGPRT$^-$) among fibroblasts cultured from obligate heterozygote mothers of affected children (79). This latter finding, which has been extended to amniotic fluid cells (80), was predicted for X-linked traits by the Lyon hypothesis of random inactivation of one of the two X-chromosomes in female cells during an early stage of development (81). Identification of heterozygous carriers of severe HGPRT deficiency is now possible by application of this concept to the study of skin fibroblasts (79) and hair follicles (82). The identification, in turn, of pregnancies at risk for the birth of affected hemizygous males has, thus, become possible. Monitoring of such pregnancies by amniocentesis and amniotic-fluid cell analysis thus provides a preventive approach for control of this serious hereditary disease. To date it has allowed prenatal diagnosis of six affected males (83–85), in four cases sufficiently early that pregnancy could be terminated (84, 85).

---

[3] Nuki, G., Astrin, K., Brenton, D., Lever, J., Seegmiller, J. E. In preparation.

HGPRT acitivty is normal in erythrocytes from obligate heterozygotes for severe HGPRT deficiency. Erythrocytes and leukocytes from two women heterozygous for both severe HGPRT deficiency and glucose 6-phosphate dehydrogenase variants showed expression of only the variant of glucose 6-phosphate dehydrogenase carried on the chromosome with the HGPRT$^+$ allele (86). Nonrandom inactivation of the X-chromosome of peripheral blood cell precursors carrying the HGPRT$^-$ allele or selection against cells carrying the HGPRT$^-$ gene following random X-chromosome inactivation are the alternative explanations suggested by these results. Evidently care is needed in selecting a source for identifying heterozygous carriers of genetic diseases and caution must be used in interpreting modes of genetic transmission from studies in hematopoietic tissues (as noted previously in the case of increased PRPP synthetase activity).

Although severe HGPRT deficiency shows X-linked recessive inheritance, many females heterozygous for severe HGPRT deficiency manifest purine overproduction and even clinical gout, particularly after the menopause (87). Thus, the possibility of the heterozygous state for severe HGPRT deficiency should be considered in females with clinical gout despite normal erythrocyte HGPRT activities.

PARTIAL DEFICIENCY OF HGPRT Less severe deficiencies of HGPRT than those characteristic of the Lesch-Nyhan syndrome are responsible for some cases of purine overproduction usually manifested by onset in adolescence or early adult life of gouty arthritis or uric acid urolithiasis. About 20% of males afflicted with these X-linked structural defects in HGPRT show neurologic abnormalities of varying types and degrees (70). While generally less disabling than in severe HGPRT deficiency, the neurologic findings in partial HGPRT deficiency are more variable: mental retardation, seizures, spasticity, and spinocerebellar syndromes have, among other findings, been reported (70). A correlation exists between magnitude of residual enzyme activity and the parameters of abnormal purine metabolism and severity of neurological dysfunction (70). Some exception to this latter association has, however, recently been noted (88).

Seven patients in three families with partial HGPRT deficiency were found among 425 patients with primary gout in a recent report (89) and only one family with increased PRPP synthetase activity has been found among 100 gouty patients screened to date.[4] These findings emphasize the small proportion (less than 10%) of gouty patients with overproduction of uric acid in whom specific enzyme defects can be identified at present.

The abnormalities of purine metabolism incident to partial HGPRT deficiency are those of the severe deficiency but are quantitatively less striking. Again, increased intracellular PRPP concentration is a consistent biochemical correlate of increased purine production in cells from these individuals (58). An interesting difference, recently confirmed, is the intermediate HGPRT activities found in erythrocytes in

[4] Becker, M. A., Meyer, L. J., Seegmiller, J. E. Unpublished data.

some but not all obligate heterozygotes for the partial deficiency (90). Selection in bone marrow precursors against X-chromosomes carrying certain alleles for partial HGPRT deficiency apparently does not occur. A direct relationship between the erythrocyte PRPP concentration and the degree of deficiency of HGPRT activity was found in these heterozygous women (90).

PARTIAL APRT DEFICIENCY In all four of the reported families with partial APRT deficiency, activities of the erythrocyte enzyme have been considerably less than 50% of normal (91–94). The deficiency appears to be inherited autosomally (95) and reported cases probably represent heterozygous deficiency states. Inactivation of a dimeric enzyme by a mutant subunit could account for the markedly decreased enzyme activity found in the heterozygote (95). The normal active enzyme appears to consist of 11,000 dalton subunits arranged in dimers, trimers, and hexamers (96).

The coexistence of gout with partial APRT deficiency in three families (92–94) may merely reflect the screening only of gouty populations. A direct relationship between the enzyme deficiency and gout remains to be proven. In two families the enzyme defect and gout segregate separately in the pedigrees (92, 94). In the third, erythrocyte PRPP values and urate production are normal (93). Although severe APRT deficiency has not yet been described, the clinical expression to be expected from the defect includes a hemolytic or macrocytic anemia from impaired formation of adenine nucleotides or gout with overproduction of uric acid from either an analogous increase in PRPP or diminished feedback inhibition of PRPP amidotransferase.

Altered Feedback Inhibition

Modulation of the rate of purine synthesis de novo by feedback inhibition of PRPP amidotransferase activity could be disrupted in several ways. Increases in PRPP availability have already been discussed. Diminished concentrations of one or more inhibitory purine nucleotides represent another mechanism for activating this pathway. While purine nucleotide depletion may lead to the increased rate of purine synthesis de novo that results from rapid infusion of fructose in man,[5] no evidence has yet been found for such a mechanism in known hereditary aberrations in purine synthesis. In fact, normal purine nucleotide pool sizes have been demonstrated in HGPRT-deficient fibroblasts (53) and lymphoblasts,[3] thus ruling out one proposed mechanism for purine overproduction with this enzyme defect.

A genetically altered PRPP amidotransferase which is relatively insensitive to feedback inhibitors constitutes a third potential alteration leading to overproduction of purines. Fibroblasts cultured from two unrelated patients with gout and marked overproduction of uric acid showed a diminished responsiveness of the purine synthetic rate to inhibition by purine bases. On this basis an abnormality in response of PRPP amidotransferase to feedback inhibition has been postulated (59). The fibroblasts of both patients show increased PRPP concentration and subsequently, in the cells of one patient, increased PRPP generation and increased PRPP synthe-

[5] Raivio, K. O., Becker, M. A., Seegmiller, J. E. In preparation.

tase activity were demonstrated (22). No abnormality in PRPP synthetase has yet been found in the other patient, however, despite extensive study.[4] The recent development of a method for direct study of human PRPP amidotransferase in lymphoblasts (97) may help to clarify the problem in this patient.

OTHER POTENTIAL DISORDERS OF PURINE METABOLISM

Two metabolic accompaniments of purine overproduction deserve mention. First, activity of xanthine oxidase measured in liver material obtained at needle biopsy was increased about fourfold in each of eight gout patients with excessive uric acid excretion (98). In fact, when normal individuals were made hyperuricemic by the oral administration of RNA or hypoxanthine for several days, hepatic xanthine oxidase activity was also increased significantly (99). Thus, the possibility of secondary differences in enzyme activity resulting from primary genetic alterations in other steps of metabolism must always be considered. Secondary increases in activity of APRT (100), inosinic acid dehydrogenase (101), orotate phosphoribosyltransferase, and orotidylic acid decarboxylase (102) have been observed in erythrocytes of patients with severe deficiency of HGPRT.

The second metabolic accompaniment of excessive purine metabolism of gouty subjects was originally interpreted as a primary aberration of glutamine metabolism. Patients with excessive uric acid synthesis showed a disproportionately large incorporation of ^{15}N from administered ^{15}N-glycine into the N_3 and N_9 atoms of uric acid arising from glutamine (103). Gutman & Yü proposed that this aberrant pattern of labeling reflected an increased concentration of intracellular glutamine, which in turn was responsible for the excessive rate of uric acid synthesis in gout (104). They further proposed that both the hypothetical increase in glutamine concentration and a previously observed deficit in urinary ammonia excretion by these patients (105) resulted from a primary deficiency in renal glutaminase (104). While providing an attractive unifying theory to explain gout, much evidence has been found to refute the "glutamine hypothesis." First, normal activities of phosphate-activated glutaminase, pyruvate-activated glutaminase, and nonactivated glutaminase have been found in renal tissue from gouty patients (106). Second, plasma glutamine concentrations are normal in these individuals (107). Third, the deficit in renal ammonia excretion, which is an age-related phenomenon accelerated by many renal diseases, correlates with the extent of preceding renal tubular damage rather than with a primary enzymatic deficiency (108). Finally, study of the kinetics of intramolecular distribution of ^{15}N in uric acid after ^{15}N-glycine administration to patients in whom excessive purine synthesis is clearly attributable to increased PRPP availability demonstrates that the preferential enrichment of N_3 and N_9 of synthesized uric acid is dependent on accelerated purine biosynthesis per se rather than a specific defect in glutamine metabolism (48, 109).

Recently, a modification of the glutamine hypothesis has been formulated that takes into account the increased plasma glutamate levels found early in association with hyperuricemia (110, 111). A deficit in glutamate catabolism perhaps related to diminished activity of glutamic acid dehydrogenase has been substituted for glutaminase deficiency. Implicit in this hypothesis once again is the increased availability of glutamine to drive hepatic purine synthesis. While under certain carefully regulated experimental conditions glutamine concentration does exert a regulatory role in purine synthesis de novo (112), evidence to date does not support a major physiologic regulatory role for this amino acid. Kinetic studies of the PRPP amidotransferase reaction (39) suggest that at the concentration of glutamine normally found in plasma (107) this enzyme is saturated with glutamine, making further increases in glutamine concentrations unlikely to stimulate purine synthesis de novo.

Literature Cited

1. Neuwirth, E. 1943. *Arch. Intern. Med.* 72:377–87
2. Garrod, A. B. 1848. *Trans. Med. Chir. Soc. London* 31:83–98
3. Garrod, A. E. 1931. *The Inborn Factors in Disease: An Essay.* 106–11. Oxford: Clarendon. 160 pp.
4. Garrod, A. E. 1908. *Lancet* 2:1–7, 73–79, 142–48, 214–20
5. Smyth, C. J. 1957. *Metabolism* 6: 218–29
6. Smyth, C. J., Cotterman, C. W., Freyberg, R. H. 1948. *J. Clin. Invest.* 27: 749–59
7. Stecher, R. M., Hersh, A. H., Solomon, W. M. 1949. *Ann. Intern. Med.* 31: 595–614
8. Hauge, M., Harvald, B. 1955. *Acta Med. Scand.* 152:247–57
9. Neel, J. V., Rakic, M. T., Davidson, R. T., Valkenburg, H. A., Mikkelsen, W. M. 1965. *Am. J. Hum. Genet.* 17:14–22
10. Benedict, J. D., Forsham, P. H., Stetten, DeW. Jr. 1949. *J. Biol. Chem.* 181: 183–93
11. Benedict, J. D. et al 1952 *Metabolism* 1:3–12
12. Benedict, J. D., Yü, T. F., Bien, E. J., Gutman, A. B., Stetten, DeW. Jr. 1953. *J. Clin. Invest.* 32:775–77
13. Wyngaarden, J. B. 1957. *J. Clin. Invest.* 36:1508–15
14. Wyngaarden, J. B. 1958. *Metabolism* 7:374–75
15. Seegmiller, J. E., Laster, L., Liddle, L. V. 1958. *Metabolism* 7:376–77
16. Sorenson, L. B. 1960. *Scand. J. Clin. Lab. Invest.* 12: Suppl. 54. 214 pp.
17. Seegmiller, J. E., Grayzel, A. I., Laster, L., Liddle, L. V. 1961. *J. Clin. Invest.* 40:1304–14
18. Alepa, F. P., Howell, R. R., Klinenberg, J. R., Seegmiller, J. E. 1967. *Am. J. Med.* 42:58–66
19. Seegmiller, J. E., Rosenbloom, F. M., Kelley, W. N. 1967. *Science* 155: 1682–84
20. Kelley, W. N., Rosenbloom, F. M., Henderson, J. F., Seegmiller, J. E. 1967. *Proc. Nat. Acad. Sci. USA* 57:1735–39
21. Sperling, O., Boer, P., Persky-Brosh, S., Kanarek, E., de Vries, A. 1972. *Rev. Eur. Etud. Clin. Biol.* 17:703–6
22. Becker, M. A., Meyer, L. J., Wood, A. W., Seegmiller, J. E. 1973. *Science* 179: 1123–26
23. Alvsaker, J. O. 1968. *J. Clin. Invest.* 47:1254–61
24. Klinenberg, J. R., Kippen, I. 1970. *J. Lab. Clin. Med.* 75:503–10
25. Schulman, J. D., Lustberg, T. J., Kennedy, J. L., Museles, M., Seegmiller, J. E. 1970. *Am. J. Med.* 49:118–24
26. Goldfinger, S., Klinenberg, J. R., Seegmiller, J. E. 1965. *N. Engl. J. Med.* 272:351–55
27. Praetorius, E., Kirk, J. E. 1950. *J. Lab. Clin. Med.* 35:865–68
28. Greene, M. L., Marcus, R., Aurbach, G. D., Kazam, E. S., Seegmiller, J. E. 1972. *Am. J. Med.* 53:361–67
29. Simkin, P. A., Skeith, M. D., Healey, L. A. 1973. *Isr. J. Med. Sci.* 9:34
30. Bishop, C., Zimdahl, W. T., Talbott, J. H. 1954. *Proc. Soc. Exp. Biol. Med.* 86:440–41
31. Wallis, L. A., Engle, R. L. 1957. *Am. J. Med.* 22:13–23
32. Kelley, W. N., Rosenbloom, F. M., Miller, J., Seegmiller, J. E. 1968. *N. Engl. J. Med.* 278:287–93
33. Popert, A. J., Hewitt, J. V. 1962. *Ann. Rheum. Dis.* 21:154–63
34. Scott, J. T., Pollard, A. C. 1970. *Ann. Rheum. Dis.* 29:397–400
35. Seegmiller, J. E. 1965. *Arthritis Rheum.* 8:714–23
36. Hall, A. P., Barry, P. E., Dawber, T. R.,

McNamara, P. M. 1967. *Am. J. Med.* 42:27–37

37. Weissmann, G., Rita, G. A. 1972. *Nature (New Biol.)* 240:167–72
38. Seegmiller, J. E., Howell, R. R. 1962. *Arthritis Rheum.* 5:616–23
39. Holmes, E. W., McDonald, J. A., McCord, J. M., Wyngaarden, J. B., Kelley, W. N. 1973. *J. Biol. Chem.* 248: 144–50
40. Fox, I. H., Kelley, W. N. 1971. *Ann. Intern. Med.* 74:424–33
41. Holmes, E. W. Jr., Wyngaarden, J. B., Kelley, W. N. 1973. *J. Biol. Chem.* 248:6035–40
42. Wyngaarden, J. B., Ashton, D. M. 1959. *J. Biol. Chem.* 234:1492–96
43. Caskey, C. T., Ashton, D. M., Wyngaarden, J. B. 1964. *J. Biol. Chem.* 239: 2570–79
44. Nierlich, D. P., Magasanik, B. 1965. *J. Biol. Chem.* 240:358–65
45. Fox, I. H., Wyngaarden, J. B., Kelley, W. N. 1970. *N. Engl. J. Med.* 283: 1177–82
46. Emmerson, B. T. 1966. *Ann. Rheum. Dis.* 25:622
47. Schulman, J. D., Greene, M. L., Fujimoto, W. Y., Seegmiller, J. E. 1971. *Pediat. Res.* 5:77–82
48. Seegmiller, J. E., Klinenberg, J. R., Miller, J., Watts, R. W. E. 1968. *J. Clin. Invest.* 47:1193–1203
49. Kelley, W. N. et al 1970. *Metabolism* 19:1025–35
50. Kelley, W. N., Fox, I. H., Wyngaarden, J. B. 1970. *Biochim. Biophys. Acta* 215:512–16
51. Boyle, J. A., Raivio, K. O., Becker, M. A., Seegmiller, J. E. 1972. *Biochim. Biophys. Acta* 269:179–83
52. Kelley, W. N., Fox, I. H., Wyngaarden, J. B. 1970. *Clin. Res.* 18:457
53. Rosenbloom, F. M., Henderson, J. F., Caldwell, I. C., Kelley, W. N., Seegmiller, J. E. 1968. *J. Biol. Chem.* 243: 1166–73
54. Wood, A. W., Becker, M. A., Seegmiller, J. E. 1973. *Biochem. Genet.* 9:261–74
55. Jones, O. W. Jr., Ashton, D. M., Wyngaarden, J. B. 1962. *J. Clin. Invest.* 41: 1805–15
56. Hershko, A., Hershko, C., Mager, J. 1968. *Isr. J. Med. Sci.* 4:939–44
57. Sperling, O., Ophir, R., De Vries, A. 1971. *Rev. Eur. Etud. Clin. Biol.* 16: 147–51
58. Greene, M. L., Seegmiller, J. E. 1969. *J. Clin. Invest.* 48:32a
59. Henderson, J. F., Rosenbloom, F. M., Kelley, W. N., Seegmiller, J. E. 1968. *J. Clin. Invest.* 47:1511–16
60. Sperling, O., Persky-Brosh, S., Boer, P., De Vries, A. 1973. *Biochem. Med.* 7:389–95
61. Becker, M. A., Kostel, P. J., Meyer, L. J., Seegmiller, J. E. 1973. *Proc. Nat. Acad. Sci. USA.* In press
62. Yü, T. F., Sirota, J. H., Berger, L., Halpern, M., Gutman, A. B. 1957. *Proc. Soc. Exp. Biol. Med.* 96:809–13
63. Kelley, W. N., Rosenbloom, F. M., Seegmiller, J. E., Howell, R. R. 1968. *J. Pediat.* 72:488–96
64. Jakovcic, S., Sorenson, L. B. 1967. *Arthritis Rheum.* 10:129–34
65. Long, W. K. 1962. *Science* 138:991
66. Long, W. K. 1967. *Science* 155:712–13
67. Long, W. K. 1970. *Am. J. Hum. Genet.* 22:14a–15a
68. Beutler, E. 1969. *J. Clin. Invest.* 48: 1957–66
69. Paniker, N. V., Srivastava, S. K., Beutler, E. 1970. *Biochim. Biophys. Acta* 215:456–60
70. Kelley, W. N., Greene, M. L., Rosenbloom, F. M., Henderson, J. F., Seegmiller, J. E. 1969. *Ann. Intern. Med.* 70:155–206
71. Atkinson, D. E., Fall, L. 1967. *J. Biol. Chem.* 242:3241–42
72. Lesch, M., Nyhan, W. L. 1964. *Am. J. Med.* 36:561–70
73. Rosenbloom, F. M., Kelley, W. N., Miller, J., Henderson, J. F., Seegmiller, J. E. 1967. *J. Am. Med. Assoc.* 202: 175–77
74. Fujimoto, W. Y., Seegmiller, J. E. 1970. *Proc. Nat. Acad. Sci. USA* 65:577–84
75. Rubin, C. S., Dancis, J., Yip, L. C., Nowinski, R. C., Balis, M. E. 1971. *Proc. Nat. Acad. Sci. USA* 68:1461–64
76. Arnold, W. J., Meade, J. C., Kelley, W. N. 1972. *J. Clin. Invest.* 51:1805–12
77. Kelley, W. N., Meade, J. C. 1971. *J. Biol. Chem.* 246:2953–58
78. Nyhan, W. L. 1968. *Fed. Proc.* 27: 1091–96
79. Rosenbloom, F. M., Kelley, W. N., Henderson, J. F., Seegmiller, J. E. 1967. *Lancet* 2:305–6
80. Fujimoto, W. Y., Seegmiller, J. E., Uhlendorf, B. W., Jacobson, C. B. 1968. *Lancet* 2:511–12
81. Lyon, M. F. 1961. *Nature (London)* 190:372–73
82. Gartler, S. M., Scott, R. C., Goldstein, J. L., Campbell, B., Sparkes, R. 1971. *Science* 172:572–74

83. DeMars, E., Sarto, G., Felix, J. S., Benke, P. 1969. *Science* 164:1303–5
84. Boyle, J. A. et al 1970. *Science* 169: 688–89
85. Van Heeswijk, P. J., Blank, C. H., Seegmiller, J. E., Jacobson, C. B. 1972. *Obstet. Gynecol.* 40:109–13
86. Nyhan, W. L., Bakay, B., Connor, J. B., Marks, J. F., Keele, D. K. 1970. *Proc. Nat. Acad. Sci. USA* 65:214–18
87. Emmerson, B. T., Wyngaarden, J. B. 1969. *Science* 166:1533–35
88. Emmerson, B. T., Thompson, L. 1973. *Quart. J. Med.* 166:423–37
89. Yü, T. F. et al 1972. *Ann. Intern. Med.* 76:255–64
90. Gordon, R. B., Thompson, L., Emmerson, B. T. 1973. *Isr. J. Med. Sci.* 9:17
91. Kelley, W. N., Levy, R. I., Rosenbloom, F. M., Henderson, J. F., Seegmiller, J. E. 1968. *J. Clin. Invest.* 47:2281–89
92. Fox, I. H., Kelley, W. N. 1973. *Isr. J. Med. Sci.* 9:14
93. Emmerson, B. T., Gordon, R. B., Thompson, L. 1973. *Isr. J. Med. Sci.* 9:11
94. Delbarre, F., Auscher, C., Amor, B., DeGery, A. 1973. *Isr. J. Med. Sci.* 9:9
95. Henderson, J. F., Kelley, W. N., Rosenbloom, F. M., Seegmiller, J. E. 1969. *Am. J. Hum. Genet.* 21:61–70
96. Thomas, C. B., Arnold, W. J., Kelley, W. N. 1973. *J. Biol. Chem.* 248: 2529–35
97. Wood, A. W., Seegmiller, J. E. 1973. *J. Biol. Chem.* 248:138–43
98. Carcassi, A., Marcolongo, R. Jr., Marinello, E., Riario-Sforza, G., Boggiano, C. 1969. *Arthritis Rheum.* 12:17–20
99. Marinello, E., Carcassi, A., Riario-Sforza, G., Ciccoli, L., Marcolongo, R. Jr. 1969. *Reumatismo* 21:310–11
100. Kelley, W. N. 1968. *Fed. Proc.* 27: 1047–52
101. Pehlke, D. M., McDonald, J. A., Holmes, E. W., Kelley, W. N. 1972. *J. Clin. Invest.* 51:1398–1404
102. Beardmore, T. D., Meade, J. C., Kelley, W. N. 1973. *J. Lab. Clin. Med.* 81: 43–52
103. Gutman, A. B., Yü, T. F. 1963. *Am. J. Med.* 35:820–31
104. Gutman, A. B., Yü, T. F. 1963. *Trans. Assoc. Am. Physicians* 76:141–51
105. Gutman, A. B., Yü, T. F. 1965. *J. Clin. Invest.* 44:1474–81
106. Pollak, V. E., Mattenheimer, H. 1965. *J. Lab. Clin. Med.* 66:564–70
107. Segal, S., Wyngaarden, J. B. 1955. *Proc. Soc. Exp. Biol. Med.* 88:342–45
108. Sperling, O., Frank, M., De Vries, A. 1966. *Rev. Fr. Etud. Clin. Biol.* 11: 401–4
109. Sperling, O., Wyngaarden, J. B., Starmer, C. F. 1973. *Isr. J. Med. Sci.* 9:36
110. Pagliara, A. S., Goodman, A. D. 1969. *N. Engl. J. Med.* 281:767–70
111. Gutman, A. B., Yü, T. F. 1973. *Am. J. Med.* 54:713–24
112. Raivio, K. O., Seegmiller, J. E. 1973. *Biochim. Biophys. Acta* 299:283–92
113. Chan, T.-S., Ishii, K., Long, C., Green, H. 1973. *J. Cell. Physiol.* 81:315–22

Copyright 1974. All rights reserved

2,3-DPG AND ERYTHROCYTE OXYGEN AFFINITY[1]

◆7092

George J. Brewer, M.D.

Department of Human Genetics and Department of Medicine (Simpson Memorial Institute), University of Michigan, Ann Arbor, Michigan

The discovery that 2,3-DPG profoundly influences hemoglobin oxygen affinity (1, 2) was remarkable from two standpoints. First, because of its potential importance, and second, because of the length of time (in retrospect) before the phenomenon was recognized. With respect to the first point, the linkage of hemoglobin function to red cell metabolism has profound implications for hemoglobin and red cell evolution, oxygen transport regulation, and perhaps for therapeutic intervention in a variety of diseases. With respect to the second, it was obvious for decades that oxygen affinity was inexplicably different in hemoglobin solutions compared to intact red cells, and it is surprising that the organic phosphates of the red cell were not tested earlier for effects on hemoglobin oxygen affinity. Much work is now progressing on a number of fronts of red cell metabolism and function. In this chapter we will briefly review current developments and thinking in some of these areas.

---

[1] Abbreviations used in this article are as follows: ATP and ADP, adenosine triphosphate and diphosphate, respectively; ALD, fructoaldolase; DHAP, dihydroxyacetone phosphate; 1,3-DPG, 1,3-diphosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate; DPGM, diphosphoglycerate mutase, DPGP, diphosphoglycerate phosphatase; En, enolase; FDP, fructose, 1,6-diphosphate; F-6-P, fructose 6-phosphate; GAPD, glyceraldehyde-3-phosphate dehydrogenase; G-1,6-DP, glucose 1,6-diphosphate; G-1-P, glucose 1-phosphate; G-6-P, glucose 6-phosphate; G-3-P, glyceraldehyde 3-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GSSG and GSH, oxidized and reduced glutathione, respectively; HK, hexokinase; LDH, lactate dehydrogenase; NADP and NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate, respectively; NAD and NADH, oxidized and reduced nicotinamide adenine dinucleotide, respectively; PEP, phosphoenol pyruvate; PFK, 6-phosphofructokinase; 6-PG, 6-phosphogluconate; 6PGD, 6-phosphogluconate dehydrogenase; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; PGI, phosphohexose isomerase (also called PHI); PGK, 3-phosphoglycerate kinase; PGM, phosphoglucomutase, PGYM, 2,3-phosphoglycerate mutase; P_i, inorganic phosphate; PK, pyruvate kinase; TA, transaldolase; TK, transketolase; TPI, triosephosphate isomerase.

UNIVERSITY COLLEGE OF MEDICINE
LIBRARY
C. U.

Throughout this chapter we will refer frequently to hemoglobin and red cell oxygen affinity. For orientation (Figure 1), the reader should equate a decrease in oxygen affinity with a shift to the right of the oxygen dissociation curve and an increase in *p*50.

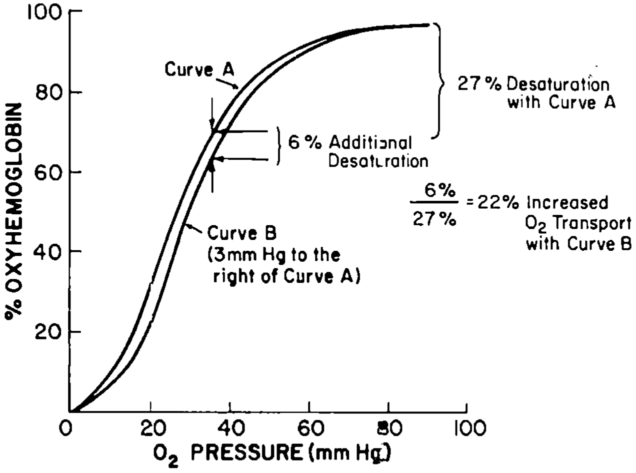


Figure 1 This figure illustrates the human red cell oxygen dissociation curve for orientation and for comparison of a normal curve (curve *A*) and a right-shifted curve (curve *B*). The calculations involve the comparisons of the two curves and are pertinent to the section of this chapter entitled "Significance of Variation in Oxygen Affinity." For orientation, the red cell oxygen dissociation curve relates the percentage of oxygen saturation of hemoglobin (% oxyhemoglobin) to oxygen pressure. The position of the dissociation curve is often evaluated by a number called the *p*50. The *p*50 is the pressure of oxygen in millimeters of mercury at which hemoglobin or erythrocytes are half saturated with oxygen. Reprinted with permission from Academic Press.

THE ROLE OF DPG AND OTHER FACTORS IN OXYGEN AFFINITY

Four factors are known to have a major influence on hemoglobin and red cell oxygen affinity (recently reviewed by Brenna et al, 3). These are pH, carbon dioxide (CO_2), temperature, and certain organic phosphates. A decrease in pH decreases oxygen affinity through the Bohr effect. An increase in CO_2 tension decreases oxygen affinity through the Bohr effect, but also because of direct combination of CO_2 in the form of carbamate with hemoglobin. An increase in temperature decreases oxygen affinity (4). Finally, organic phosphates of the erythrocyte, DPG being the most important quantitatively, decrease oxygen affinity. An effect of hemoglobin concentration on oxygen affinity has also been suggested (5), but this effect appears to be so minor as to be physiologically unimportant (6). An effect of zinc on increasing hemoglobin oxygen affinity has been recently observed (7). It is too early to know whether physiological and pathological variations in zinc levels affect erythrocyte oxygen affinity.

The mechanism of the DPG effect on oxygen affinity appears to involve binding of DPG in the central cavity of deoxyhemoglobin. According to the model of Perutz (8, 9), DPG binds to the basic hemoglobin groups valine-1, lysine-82, and histidine-143 of the two β chains. Competition occurs between DPG and CO_2 for binding sites because both bind to the N-terminal valines of the β chains of hemoglobin. Binding of DPG to hemoglobin forces the conformation of the hemoglobin molecule to the deoxy form, driving off oxygen. On the other hand, increasing oxygen tension increases oxygenation of the molecule and tends to drive off DPG. Oxyhemoglobin does not bind DPG (of physiological importance) because the stereochemistry of the central cavity of oxyhemoglobin does not allow binding.

The greatly decreased oxygen affinity of human erythrocytes (*p*50 of about 26 mm of mercury) compared to hemoglobin (*p*50 of about 5 mm of mercury) is to a major extent due to the large concentrations of DPG in human erythrocytes. Some of the other organic phosphate compounds of the red cell, such as ATP, also have this effect, but from the quantitative standpoint DPG is the most important. The erythrocytes of many other mammalian species also contain DPG, although some, such as sheep and goats, have hemoglobins with appropriate oxygen affinity and do not utilize intracellular allosteric effectors of hemoglobin such as DPG to any great extent. Birds utilize a different organic phosphate, inositol pentaphosphate, for intracellular modulation of hemoglobin oxygen affinity.

The quantitative relationship between DPG and hemoglobin oxygen affinity is such that each 0.4 m*M* increase in DPG levels decreases oxygen affinity by about 1.0 mm of mercury (10). Since the average normal level of DPG in human erythrocytes is about 5 m*M*, an increase of DPG of about 8% is required for each 1.0 mm change in *p*50.

FACTORS INFLUENCING LEVELS OF ERYTHROCYTE DPG

Individual Hemoglobin Level

Considerable variation in DPG levels, hemoglobin levels, and oxygen affinity occurs in the normal human population. A negative correlation has been consistently observed between hemoglobin levels and levels of DPG in normal people (11, 12). While the correlation between DPG and hemoglobin levels is not proven to be causal, it has been suggested that the blood of normal individuals is programmed for appropriate oxygen delivery by adjustments in the levels of one variable to compensate for the other (11, 12). That is, in individuals with relatively low (or high) hemoglobin levels the red cell metabolic mechanism may be programmed for higher (or lower) levels of DPG, while individuals with relatively low (or high) levels of DPG, for genetic or other reasons, may be hematologically programmed for a higher (or lower) level of circulating erythrocytes. This thinking suggests that DPG levels are finely tuned to oxygen transport needs in conjunction with the level of circulating erythrocytes in normal humans.

Hypoxia

Hypoxic stress of almost any type usually produces an increase in levels of DPG (12). This includes altitude exposure, anemia, heart failure, and some cases of pulmonary disease. Some patients with pulmonary disease do not have elevations of DPG, perhaps because of respiratory acidosis. The increase in DPG seen in these hypoxic conditions is primarily responsible for the decrease in erythrocyte oxygen affinity, which has been known for decades (13, 14).

Erythrocyte Glycolysis

In order to understand the mechanisms underlying changes in levels of DPG and the accompanying changes in oxygen affinity, it is essential to understand control of red cell glycolysis since DPG is a glycolytic product. Ordinarily the rate of glycolysis in the red cell is under constant inhibition through limitation of the activity of certain rate-controlling enzymes (15–17). The mature red cell is not capable of protein synthesis, so acute (hours to weeks) changes in glycolysis must take place through activation (or decreased inhibition) of the activity of these rate-limiting enzymes. Considerable progress in understanding glycolytic control has been made by Yoshikawa & Minakami (15). Based on assays of glycolytic intermediates and free energy changes at various steps of glycolysis, it appears that control is exercised primarily at the HK, PFK, and PK enzymatic steps (15). In chronic (many weeks to months) hypoxia the number of enzyme molecules incorporated into the cell in the bone marrow may also change and thereby affect glycolysis. Another factor influencing the level and activity of red cell enzymes is the mean age of the red cell population, since many red cell glycolytic enzymes are age-dependent. Thus, young red cell blood cells tend to have higher activities of most glycolytic enzymes.

Many possible mechanisms for changing the levels of DPG in the red cell are possible (Figure 2). These include an overall increase in glycolytic rate, an increased conversion of 1,3-DPG to 2,3-DPG, a decreased degradation of 2,3-DPG to 3-PG, a block in the pathway below 3-PG, a change in the rate of flow through the pentose phosphate pathway, or combinations of these various mechanisms. An additional complexity is that once an increase in flow either through the glycolytic or pentose phosphate pathway is established, previously non-rate-limiting steps of the pathway may then become rate-limiting.

On the basis of various lines of evidence it appears at present that an increase in overall glycolytic rate as controlled by HK, PFK, and possibly PK is probably the major factor in producing elevations of DPG in hypoxia, with effects on the enzymes of the DPG shunt (DPGM or DPGP) as additional modulating factors (18). It is clear that an increase in pentose phosphate pathway flow can also increase DPG levels (17, 19). Another important factor appears to be limitation of the GAPD step once high glycolytic flow has occurred. A number of possible effectors of some of these enzyme steps are known and will now be briefly discussed.

PFK AND pH One important regulatory factor in red cell glycolysis is pH. As pH increases glycolysis increases. This seems to be due primarily to a relative activation of PFK, because as the pH of incubation is increased the levels of intermediates prior to the PFK step decrease, while the levels of intermediates subsequent to this step increase (20). The mechanism of the pH activation effect on PFK may be multiple in that as the pH increases, the binding of the substrate F-6-P is increased and the allosteric inhibition by ATP is decreased (16). The inhibition by ATP is also decreased by P_i (20). Other factors that may regulate PFK activity are ADP and

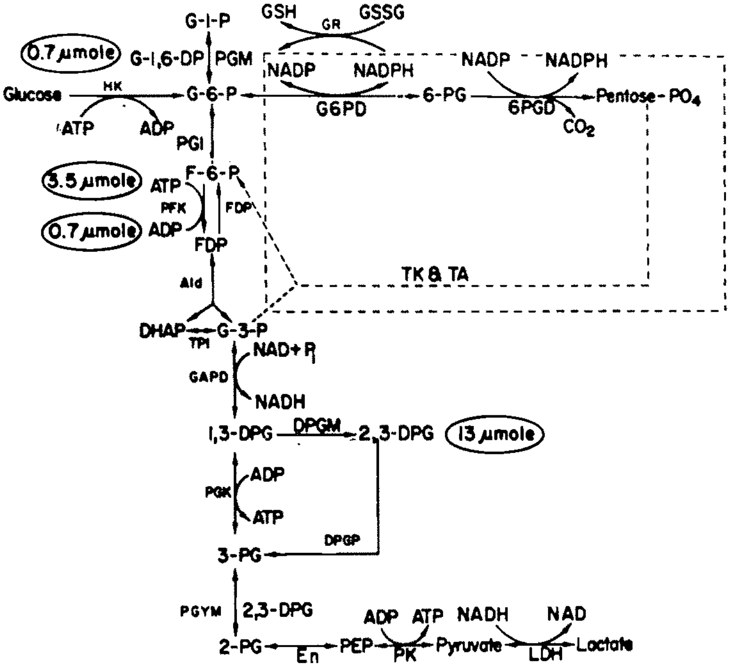


Figure 2 The major pathways of carbohydrate metabolism in mature mammalian red cells. The arrows represent enzymatic steps. The circled numbers indicate amounts, in µmole per g of hemoglobin, of phosphorylated intermediates present in highest concentrations in human erythrocytes. The pentose phosphate pathway is enclosed in the dotted lines. Modified from (12) with permission from *Science.*

Mg^{2+}. ADP is an activator and thus the ratio of ADP to ATP may play a critical role in determining the activity of the enzyme (21). MgATP is the probable substrate for PFK and is less an inhibitor than ATP (16). Thus, the level of Mg^{2+} in the cell may play an important role in the activity level of PFK.

HK Factors that influence HK activity include inhibition by its product, G-6-P (22). The enzyme may also be limited by a lack of saturation with MgATP, although the level of MgATP in the red cell is not very certain. ATP also competitively relieves the inhibition of HK by G-6-P. Thus, factors that increase either Mg^{2+} or ATP in the red cell are potential activators of HK. In addition, P_i is an important potential activator of HK since it appears to relieve the inhibition of HK by G-6-P (23).

DPG Another potentially important effector of red cell glycolysis is DPG, which inhibits certain glycolytic enzymes such as HK (24, 25). The DPG inhibition of HK is increased by decreasing concentrations of ATP and/or Mg^{2+} (25). Inhibition of rate-limiting enzymes of glycolysis by DPG provides a possible negative feedback loop by which the level of DPG partially controls itself.

P_i In view of its possible role in activating both PFK and HK, the level of serum P_i is potentially important in influencing red cell glycolytic rate. It has been shown that administration of P_i increases levels of DPG (26, 27) and that decrease in serum P_i results in decrease in levels of DPG (28). This latter result may be due primarily to limitation of the GAPD reaction with prolonged decrease in serum P_i (16). The role of P_i in regulating DPG and oxygen affinity will be further discussed in the section on therapeutic manipulation of oxygen affinity.

Assay of Glycolytic Intermediates to Identify Mechanisms of Glycolytic Stimulation

The pioneering use of assays of glycolytic intermediates of blood samples after in vitro manipulation by Minakami & Yoshikawa (15, 20) has contributed significantly to our understanding of glycolytic control. However, actual intracellular conditions during in vivo stresses or resulting from in vivo stimuli may vary greatly from conditions that are obtainable during in vitro studies. For instance, many of the in vivo adjustments require several days. For these reasons the assay of erythrocyte glycolytic intermediates in humans undergoing various hypoxic stresses can add greatly to our understanding of the enzymatic mechanisms of the red cell response to hypoxia. In some cases (e.g. altitude stress), the assay of intermediates in the same person prior to exposure may serve as the control or reference value. In other cases (e.g. anemic patients), the values of the patients must be compared to appropriate controls, such as the mean of a sample of normal individuals. Work that has been initiated in this area indicates that during both acute and chronic altitude exposure (29) and in anemia (30) HK is generally activated and PFK is frequently activated. The mechanism of the PFK activation appears to be an increase in pH, generally due to respiratory alkalosis. The mechanism of HK activation is not clear.

Genetic, Age, and Sex Effects

GENETIC EFFECTS The levels of erythrocyte DPG in an outbred strain of rat (Hooded) are under strong genetic influences (31). Genetic selection has resulted in two strains of rat, one high DPG and the other low, which breed true and which have a marked difference in *p*50 corresponding to their levels of DPG. The inheritance of DPG in man has not been studied as yet, but the situation will presumably be similar to the findings with erythrocyte DPG in rats and ATP in man, which is also under strong genetic influence, probably of a multifactorial type (32). If so, genetically determined variation in DPG may be an important factor underlying normal human variation in *p*50 and hemoglobin values. The mechanism of the genetic influence on factors such as DPG and ATP may reside in small contributions of many genes (multigenic inheritance), such as genes influencing pH, serum P_i and Mg^{2+} levels, and possibly alleles determining activity differences of the various glycolytic enzymes.

AGE EFFECTS Some evidence exists for a gradual decline in red cell metabolism with aging. Both DPG levels (26) and ATP levels (32, 33) decline somewhat with age in man, but the mechanism is unknown. The decline is most marked during childhood.

SEX EFFECTS Hemoglobin levels are lower, but DPG levels are higher (6, 34) and oxygen affinity lower (34) in adult women than in adult men. The higher DPG levels in erythrocytes of women is apparently maintained through a higher glucose consumption (35) than that of men, perhaps as a result of greater HK activity (6). Thus, the blood of women appears to be programmed for oxygen delivery in a somewhat different manner than that of men.

SIGNIFICANCE OF VARIATION IN OXYGEN AFFINITY

It can be shown theoretically (Figure 1) that decrease in oxygen affinity of the order of a 3 mm mercury change in *p*50 causes a 22% improvement in oxygen transport in resting man in the absence of changes in hemoglobin, cardiac output, ventilation, and arterial oxygen tension (18). In the example shown in Figure 1, the blood is 97% saturated in the lungs and desaturates to a tissue capillary oxygen tension of 36 mm of mercury (vertical arrows of the two curves). Curve *A* is that of red cells with a *p*50 of 26 mm of mercury. At 36 mm such red cells have reached the saturation value of 70%, which means they have lost 27% of total oxygen capacity. Curve *B*, with a *p*50 of 29 mm, loses additional oxygen amounting to 6% of total oxygen capacity in dropping to an oxygen tension of 36 mm. Thus, 6%/27% = 22% theoretical gain in oxygen delivered by curve *B* compared to curve *A*, without an increase in cardiac output or decrease in tissue capillary oxygen pressure. However, surprisingly little data have accumulated over the years to verify that such theoretical effects on oxygen transport bring about objective evidence of improved adaptation to hypoxia. It has been shown in rats that exercise tolerance is not affected by rather marked differences in level of DPG and oxygen affinity (36). However, since skeletal muscle and perhaps cardiac muscle can extract most of the oxygen from blood irrespective of oxygen affinity, it is perhaps not surprising that exercise performance is not affected by oxygen affinity. The function of organs such as the central nervous system in the presence of variation in oxygen affinity has not been tested. This is an important area for future research because methods are now becoming available to manipulate DPG levels and oxygen affinity. The usefulness of such therapy can only be evaluated if the effects of change in oxygen affinity are known.

One disease area in which it may be tentatively concluded that manipulation of oxygen affinity is useful is sickle cell anemia. Preliminary success in the treatment of sickle cell anemia with the drug cyanate (27) has opened new thinking in the treatment of this disease. Cyanate causes an increase in oxygen affinity by blocking binding of DPG to hemoglobin. Cyanate binds irreversibly to the N-termainal valines of the β and α chains of hemoglobin. One line of thinking holds that the beneficial effect of cyanate in sickle cell anemia may result from this increase in oxygen affinity, which causes the cell to resist deoxygenation. The cyanate-treated cell resists sickling because only deoxyhemoglobin will polymerize and bring about the sickling phenomenon.

THERAPEUTIC MANIPULATION OF OXYGEN AFFINITY

Stimulation of Glycolysis

Assuming that a decrease in oxygen affinity may be beneficial in various hypoxic diseases and stresses, we can briefly discuss known mechanisms for stimulating glycolysis and the production of DPG. In Rhesus monkeys Sugerman and colleagues (19) have successfully used a combination of inosine, pyruvate, and P_i to stimulate DPG levels up to 50% above baseline. Some of the possible enzymatic mechanisms of the P_i stimulation in this combination, including activation of HK and PFK, have already been discussed. The pyruvate, by virtue of its action in the LDH reaction, increases the availability of NAD for the GAPD step which, as discussed, may become limiting under situations of high substrate flow. Inosine provides pentose which enters the pentose phosphate pathway, bypassing the HK regulatory step. This combination of agents has not been used in man as yet. One problem is that uric acid is generated from inosine and administration of inosine will lead to an increase in blood uric acid.

Phosphate alone has some effect on elevating DPG levels. A single oral or intravenous dose of 30–60 mmole P_i given three times a day to humans results in a DPG level about 10–15% above baseline (27).

Inhibition of Glycolysis

The beneficial effect of cyanate on inhibiting sickling and improving the hematologic status of sickle cell patients suggests that other agents that increase oxygen affinity may be beneficial. This opens the approach of inhibiting red cell production of DPG for the treatment of sickle cell disease. One technique that deserves trial is the moderate lowering of serum P_i, since it has been shown in non-sickle cell anemic patients that this will decrease DPG levels (28) and presumably increase oxygen affinity.

CONCLUDING REMARKS

It is increasingly clear that the metabolism of the red cell, through DPG, is intimately linked to the function of the cell in oxygen transport. Considerable normal variability is present in DPG levels, *p*50, and hemoglobin levels, and this variability may or may not have an influence on health and response to stress. For instance, we have previously hypothesized that a relatively increased oxygen affinity may play a role in the pathogenesis of arteriosclerosis (38). Thus, individuals with relatively low DPG levels may be at a greater risk for arteriosclerotic disease. This would explain the association between higher hemoglobin levels and risk of coronary heart disease, since DPG levels and hemoglobin levels are negatively correlated, as discussed in an early section of the chapter.

The intimate role of red cell metabolism in oxygen transport reveals a new dimension for potential therapeutic exploitation in hypoxic stress, as has been discussed in the last section of this chapter. In addition, this new functional role of red cell metabolism has caused a renewed interest in understanding the cell's metabolic regulatory systems, a subject of considerable fascination in its own right and one which may have pertinence in a number of areas of cellular biology.

ACKNOWLEDGMENTS

I am indebted to Dr. Fred J. Oelshlegel for helpful comments during the preparation of this manuscript. I thank Academic Press for permission to reproduce Figure 1, which came from the second edition of *The Red Blood Cell* (In press), D. M. Surgenor, Ed., and I thank the editors of *Science* for permission to reproduce Figure 2 from *Science* 171:1205, 1971 (copyright 1971 by the American Association for the Advancement of Science).

Literature Cited

1. Chanutin, A., Curnish, R. R. 1967. *Arch. Biochem. Biophys.* 121:96
2. Benesch, R., Benesch, R. E. 1967. *Biochem. Biophys. Res. Commun.* 26:162
3. Brenna, O. et al 1972. *Hemoglobin and Red Cell Structure and Function,* ed. G. J.. Brewer. *Advan. Exp. Med. Biol.* 28:19–40. New York: Plenum. 526 pp.
4. Lenfant, C., Bellingham, A. J., Detter, J. C. 1972. *Alfred Benzon Symposium, Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status,* eds. M. Rørth, P. Astrup, IV: 736–49. New York: Academic. 832 pp.
5. Finch, C. A., Lenfant, C. 1972. *N. Engl. J. Med.* 286:407
6. Brewer, G. J., Oelshlegel, F. J. Jr., Schoomaker, E. B., Knutsen, C. See Ref. 3, pp. 99–119
7. Oelshlegel, F. J. Jr., Brewer, G. J., Prasad, A. S., Knutsen, C., Schoomaker, E. B. 1973. *Biochem. Biophys. Res. Commun.* 53:560
8. Perutz, M. F. 1970. *Nature* 228:726
9. Perutz, M. F., Pulsinelli, P. D., Ranney, H. M. See Ref. 3, pp. 3–18
10. Duhm, J. 1972. *Alfred Benzon Symposium, Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status,* eds. M. Rørth, P. Astrup, IV: 539–51. New York: Academic. 832 pp.
11. Eaton, J. W., Brewer, G. J. 1968. *Proc. Nat. Acad. Sci.* 61:756

12. Brewer, G. J., Eaton, J. W. 1971. *Science* 171:1205
13. Richards, D. W., Strauss, M. L. 1927. *J. Clin. Invest.* 4:105
14. Aste-Salazar, R., Hurtado, A. 1949. *Am. J. Physiol.* 142:733
15. Yoshikawa, H., Minakami, S. 1968. *Folia Haematol.* 89:357
16. Rapoport, S. 1968. *Essays in Biochemistry,* eds. P. N. Campbell, G. D. Greville, 4:69–103. New York: Academic. 233 pp.
17. Rapoport, S. 1970. *Bull. Soc. Chim. Biol.* 52:1169
18. Brewer, G. J. 1974. *The Red Blood Cell,* ed. D. M. Surgenor. Chap. 11. New York: Academic. 2nd ed. In press
19. Sugerman, H. J. et al 1972. *Blood* 39:525
20. Minakami, S., Yoshikawa, H. 1966. *J. Biochem.* 59:145
21. Mills, G. C. 1969. *Texas Rep. Biol. Med.* 27:773
22. Rose, I. A., O'Connell, E. L. 1964. *J. Biol. Chem.* 239:12
23. Rose, I. A., Warms, J. V., O'Connell, E. L. 1964. *Biochem. Biophys. Res. Commun.* 15:33
24. Dische, Z. 1941. *Bull. Soc. Chim. Biol.* 23:1140
25. Brewer, G. J. 1969. *Biochim. Biophys. Acta* 192:157
26. Brain, M. C., Card, R. T. See Ref. 3, pp. 145–54
27. Moore, L. G., Brewer, G. J., Oelshlegel, F. J. Jr., Rose, A. M. 1973. *Oxygen Transport to Tissue,* ed. H. Bicher, D. Bruley. New York: Plenum. In press
28. Travis, S. F. et al 1971. *N. Engl. J. Med.* 285:763
29. Moore, L. G., Brewer, G. J., Oelshlegel, F. J. Jr. See Ref. 3, pp. 397–413
30. Oelshlegel, F. J. Jr., Brewer, G. J., Penner, J. A., Schoomaker, E. B. See Ref. 3, pp. 377–96
31. Noble, N., Brewer, G. J. See Ref. 3, pp. 155–64
32. Brewer, G. J. 1967. *Biochem. Genet.* 1:25
33. Gross, R. T., Schroeder, E. A. R., Brounstein, S. A. 1963. *Blood* 21:755
34. Arturson, G., Robert, M. 1971. *Acta Anaesthesiol. Scand. Suppl.* 45:22
35. Brewer, G. J., Eaton, J. W., Weil, J. V., Grover, R. F. 1970. *Red Cell Metabolism and Function,* ed. G. J. Brewer. *Advan. Exp. Med. Biol.* 6:95–114. New York: Plenum
36. Wranne, B., Woodson, R., Detter, J. See Ref. 3, pp. 449–55
37. Gillette, P. N., Peterson, C. M., Manning, J. M., Cerami, A. See Ref. 3, pp. 261–78
38. Brewer, G. J. 1972. *Alfred Benzon Symposium, Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status,* eds. M. Rørth, P. Astrup, IV:629–45. New York: Academic. 832 pp.

Copyright 1974. All rights reserved

TREATMENT OF ACUTE GRANULOCYTIC LEUKEMIAS

❖7093

J. Bernard, M. Weil, and Cl. Jacquillat
Institut de Recherches sur les Maladies du Sang, Hôpital Saint-Louis, Paris, France

All authors contrast the poor prognosis of acute granulocytic leukemias (AGL) with the progress achieved in acute lymphocytic leukemias (ALL). However, many advances have already been made in the treatment of this disease: the median survival remains poor with present chemotherapy but survival has been shown to be directly related to the attainment of a complete remission. The indications for such an intensive treatment must be individualized according to the age of the patient and the morphology of his leukemia.

Cytological classification of acute granulocytic leukemias is difficult. The cytology of acute promyelocytic and acute monoblastic leukemia are now well characterized but the term acute myeloblastic leukemia still includes various cytological variants.

We shall deal first with the treatment of acute myeloblastic leukemia (AML) with an overt maturation arrest (high rate of poorly differentiated cells); then we shall consider the specific problems raised by acute promyelocytic leukemias (APL) and acute monoblastic leukemias (AMoL), and the difficulties encountered in the treatment of the heterogeneous "partial" leukemias.

TREATMENT OF TYPICAL ACUTE MYELOBLASTIC LEUKEMIA

Induction Treatment

Table 1 summarizes the effective drugs and their activities. In particular, we shall consider recent data on cytosine arabinoside (Ara C), daunorubicin (DNR) and its parent compounds, and combination therapy: some of these studies have provided information on specific drug resistance and may open new avenues to chemotherapy.

CYTOSINE ARABINOSIDE (ARA C) The initial proposed dose for Ara C was 30 mg/m², and there seemed to be no difference whether the dose was delivered during 24 or 4 hr (4). It now seems that the best results are obtained with injections over 120 hr at 100 mg/m²/day or with doses every 8 hr for 4 days each fortnight, the dose varying from 25 mg/m² to 120 mg/m².

Table 1 Drugs active in AML

	Percentage of Complete Remissions	Activity	Toxicity	References
Prednisone	10%	1	1	BMRC (1)
6-Mercaptopurine	10%	1	1	Frei (2)
Methotrexate	10%	1	1	Frei (2)
Methyl-Glyoxal Guanyl-Hydrazone	15%	1	2	Levin (3)
Cytosine arabinoside	20% 30%	3	1	Ellison (4) Bernard J. (5)
Daunorubicin	50% 30% 50%	4	2	Boiron M. (6) ALGB (7) Wiernick (8)
Rubidazone	50%	4	2	Jacquillat (9)
Adriamycin	6%	1	2	Cortes (10)

A requirement for the action of this drug is prior nucleotide conversion (11, 12). Competition between Ara C triphosphate and deoxycytidine triphosphate for DNA polymerase seems to be the principal nature of the drug's action. In man, Ara C is also rapidly deaminated to produce arabinosyl uridine, which has no therapeutic activity, and it should be noted that both serum and liver contain pyrimidine nucleoside deaminase (13). Consequently, the half-life of unaltered Ara C in humans is brief (about 15 min). These characteristics explain why Ara C is so specific to the S phase of the cell cycle and why its administration should be continuous or frequent.

At the cellular level, Kessel, Hall & Rosenthal (14) showed a correlation between the capacity for Ara C phosphorylation and sensitivity to the drug. The level of Ara C phosphorylation is high in sensitive AML cells and lymphocytes, intermediate in normal polynuclears, and lowest in resistant AML cells.

On the other hand, Steuart & Burke (15) showed that leukemic cells in responders contained six times less cytidine deaminase than can be found in the cells of nonresponders. The sensitivity of patients with leukemias or other malignant diseases to Ara C thus depends on the intracellular ratio of Ara C phosphorylase and Ara C deaminase; this ratio decreases when resistance occurs.

DAUNORUBICIN (DNR) DNR was the first drug which, when used alone, induced complete remission (CR) in almost 50% of cases (6, 8, 16).

Dosage schedule is based upon the necessity of achieving bone marrow aplasia, which is a prerequisite for complete remission, as soon as possible: the best results are obtained with daily doses of 2 mg/kg (60 mg/m^2). The total amount of drug necessary is 600 mg/m^2. There is, however, a marked divergence from the median.

The number of doses is actually adjusted using the curve of circulating blood cells, the decrease of blastic cells, and bone marrow cellularity. Aplasia generally begins about the fifth day after the onset of treatment and persists for two to three weeks or more. The prognosis thus depends on the hazards of this period and on the speed of regeneration of differentiated cells. During this phase, when white blood cells reappear in the blood, repeated bone marrow aspirations are necessary in order to discriminate between the two types of evolution that have opposite therapeutic consequences: progressive regeneration of differentiated cells where no further doses are required, and repopulation with blastic cells where two or three additional doses should be given. Hoping for better results with diminished toxicity, we devised a protocol for Acute Leukemia Group B (ALGB 6706 A) in which 60 mg/m^2 was administered weekly or biweekly; results were inferior to those of the continuous schedule (7). More recently, Greene et al (17) treated 23 patients with massive intermittent doses, the initial one being 180 mg/m^2; one or two additional and usually reduced doses (120 mg/m^2) were generally necessary. In 16 patients the rate of complete remission was only 31%. These authors correlated the concentration of daunorubicin reductase (DNR reductase) in leukemic cells with its therapeutic activity and found that the concentration was lowest in patients over 60 years old.

Huffman & Bachur (18) showed also that myeloblasts from leukemic patients had nine times more DNR reductase than polynuclears from normal patients; since normal myeloblasts were not tested it cannot be assessed whether this finding is common to proliferative cells or specific to leukemic cells; because of its prolonged plasma half-life, daunorubicinol, the product of DNR reductase activity, is probably a major factor in the therapeutic activity of DNR.

DNR cardiac toxicity has been reported in previous studies by us and others (19–21), and its administration should be avoided in patients with a history of cardiac disease or who are more than 70 years old. In patients between 60 and 70 years of age the indication for DNR depends on the cardiovascular status of each patient. In other patients, the cumulative dose should not exceed 35 mg/kg; the concentration of drug in time should also be considered and the ratio of dose (mg/m^2) to months of administration should not exceed one. If these limits are respected, cardiac toxicity is not of major clinical importance in acute granulocytic leukemias.

RUBIDAZONE (RP 22,050) AND ADRIAMYCIN (NSC 123,127) Rubidazone, the benzoyl hydrazone chlorhydrate of daunorubicin (Figure 1), is active in acute myeloblastic leukemia (9). It was given by intravenous infusion in saline at a daily dose of 3–5 mg/kg until the total dose reached 20 mg/kg. Blood counts were made daily and bone marrow counts on the 5th, 10th, 15th, 20th, and 25th days. Bone marrow aplasia was usually noted after the 10th day, and if blast cells then persisted, even though white cells might be 1000/mm^3 or fewer, additional doses of RP 22,050 at 3 mg/kg were given. The results of treatment are summarized in Table 2. Of 100 patients, 53 (53%) had a complete remission. Among the 64 patients in their first attack who were previously untreated, 37 (58%) achieved complete remission. Among the 25 patients in their first relapse, 13 (48%) achieved complete remission.

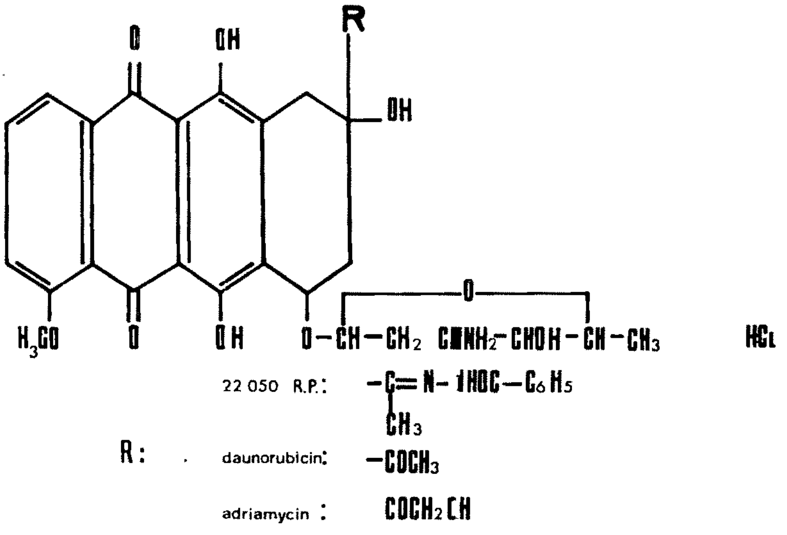


Figure 1 The chemical structure of RP 22,050.

The three patients in their second relapse were in the terminal phase of their illness. Two were resistant to RP 22,050, and one died in aplasia. The median dose needed to achieve complete remission was 23 mg/kg. In five patients, 14 mg/kg was enough, but in one patient more than 30 mg/kg was needed. These doses were given over periods varying from 10 to 30 days. The median period before blast cells completely disappeared and normal hemopoietic cells appeared was 25 days. In most cases, however, the bone marrow remained hypoplastic and the return of normal platelet counts was delayed; thus the criteria for complete remission were not fulfilled until between the 30th and the 40th day. When compared to DNR, rubidazone is less brutal in its actions and the effective dose may be given over a longer period of time, thus allowing easy individualization of the dose; it is notable that few patients died during an aplastic phase and that resistance rather than aplasia was the main cause of failure; this may be because of advances in supportive therapy and the availability of white blood cell transfusions, but it is also because the dosage of rubidazone is more easily adaptable.

Another derivative of daunorubicin, adriamycin (hydroxydaunorubicin), was isolated in 1967 by the Farmitalia Research Laboratory. Preliminary pharmacological studies (22) showed that it has a higher therapeutic index than daunorubicin. We have used adriamycin in 16 cases of acute leukemia: 2 in the first attack and 14 in relapse attacks. It induced two short remissions (one month and three months respectively) in three patients suffering their first relapse, but was ineffective in the other 11 patients in relapse. Four patients developed tachycardia and there was one case of cardiac failure.

Table 2 Results of induction in AML with rubidazone (RP 22,050)

	First Attack				First Relapse		Second Relapse		Total and %
	No Treatment		Previous Treatment						
	Children	Adults	Children	Adults	Children	Adults	Children	Adults	
Complete Remission	16	21	2	–	4	9	1	–	53 53%
Partial Remission	1	2	1	–	–	–	–	–	4 4%
Failure	5	5	1	1	4	4	2	–	22 22%
Death in Induction	1	13	–	1	–	4	–	2	21 21%
Total	23	41	4	2	8	17	3	2	100 100%

Table 3 Combination therapy in AML

Drug, Dosage, and Schedules[a]	Number of Patients	Number and Percentage of Complete Remissions	References
Ara C 50 mg/m^2 + Cycl. 50 mg/m^2 q 8 hr for 4-day courses	32	13 (41%)	Freireich (24)
Ara C 2–3 mg/kg/day + TG 2.5 mg/kg/day	36	19 (39%)	Gee (25)
Ara C 100 mg/m^2/day + TG 2.5 mg/kg/day	81	32 (40%)	Carey (ALGB) (26)
Ara C 2–3 mg/kg q 12 hr + TG 2.5 mg/kg q 12 hr	27	18 (67%)	Dowling (27)
Ara C 100 mg/m^2/day + DNR 45 mg/m^2 days 1, 2, 10, and 11	63	14 (22%)	Carey (ALGB) (26)
Ara C 2 mg/kg/day days 1, 5 + DNR 1.5 mg/kg day 1 (with or without asparaginase)	60	36 (60%)	Crowther (28)
Ara C 100 mg/m^2 days 1, 5 + DNR 100 mg/m^2 days 6, 7, and 8	22	12 (54%)	Gluckman (29)
Ara C 2 mg/kg/day x 3 + DNR 1 mg/kg/day x 3 + VCR 1.5 mg days 1 and 4	21	10 (48%)	Rosenthal (30)
Ara C 50 mg/m^2 q 8 hr days 1, 4 + Cycl. 50 mg/m^2 days 1, 4 + VCR 2 mg/m^2 day 1 + P 200 mg/m^2 days 1, 4	19	10 (53%)	Whitecar (31)

[a] Drug abbreviations: DNR: daunorubicin; Ara C: cytosine arabinoside (Cytosan®, Upjohn); Cycl.: cyclophosphamide (Cytoxan®, Mead Johnson); TG: 6-thioguanine (Thioguanine®, Burroughs-Wellcome); VCR: vincristine (Oncovin®, Lilly); P: prednisone.

Cortes, Ellison & Yates (10) reported only one remission in 15 adults with AML treated with adriamycin, whereas Mathé et al (23) reported six complete remissions in 18 cases of previously treated AML.

Our experience with rubidazone is thus far more encouraging. Its mode of action is probably near to that of DNR, but it has less hematological toxicity; its metabolism is unknown, and its probable cardiac toxicity has not yet been proven.

COMBINATION THERAPY AND SUPPORTIVE THERAPY Table 3 summarizes the main published data. Combination of DNR and Ara C was used by several authors in various sequences: DNR on day one followed by Ara C on days one to five (28), or Ara C on days one to five followed by DNR on days six, seven, and eight (29). Results are approximately equivalent and addition of asparaginase was not beneficial. The combination of Ara C and 6-thioguanine (TG) was originally proposed by Gee (25), whose regimen required daily administration of TG at 2.5 mg/kg/day orally, and Ara C, 3 mg/kg/day intravenously, during a period of 8–13 days with discontinuation of therapy upon achievement of bone marrow aplasia. In his first 36 cases, Gee obtained 19 complete remissions with a mean survival of 13 months. Similar results were obtained by ALGB (26) using an identical regimen, with 48 favorable responses observed out of a total of 85 treated patients. Using a similar protocol (ALGB 6922), less favorable results have been obtained subsequently, with only 25% complete and partial remissions. Still more recently, Clarkson (32) reported a significant increase in the effectiveness of the Ara C-TG combination when the drugs were given simultaneously at 12-hr intervals, rather than daily. Using this bidaily schedule, he observed a rate of remission twice that obtained with any previous therapy in their institutions, including once-daily Ara C-TG.

Our own experience from 1968 to 1971 is summarized in Table 4. On the whole, we achieved 48% CR in 159 adults with AML.

It thus appears that the percentage of complete remissions either with DNR or Ara C in combination with TG and other cytostatics varies considerably between different medical centers. In general, better results are achieved by investigators who have a special interest in a new approach, whereas less favorable results are obtained when the same treatment is applied in a control study. The difference may depend, at least in part, upon the extent and quality of supportive therapy.

In AML the hazard of bone marrow aplasia secondary to chemotherapy must be emphasized and a decision to commence treatment, especially with DNR and its derivatives, implies the acceptance of this risk. Whereas platelet transfusions have effectively controlled thrombocytopenic hemorrhages, granulocytopenia with its associated high incidence of infection remains a major problem. We shall not discuss here the protective sterile environment, which is not at our own disposal, nor the prophylactic use of nonabsorbable antibiotics (33), but we shall indicate our experience with leucocyte transfusions. Initially, leucocytes from patients with CML were used (34–36); we performed 1457 transfusions from 229 patients with CML in 748 cases of acute leukemia, and 62% of the patients thus treated for bone marrow aplasia improved as judged by rise in white cell counts and decrease in temperature. Results were better in children but were not influenced by the ABO Rh compatibility between donors and recipients. Today granulocytes are also obtained from

Table 4 Induction results in adults with AML (Saint-Louis 1968–1971)

Drug, Dosage, and Schedule[a]	Number of Patients	Number and Percentage of Complete Remissions	Number of Partial Remissions	Resistances	Deaths
Asparaginase 1000 U/kg/day days 1, 8 DNR 60 mg/m^2/day days 9, 10, 11	61	28 (46%)	2	12	19
Asparaginase 1000 U/kg/day days 1, 10 DNR 60 mg/m^2/day days 1, 2, 3	24	13 (54%)	2	5	4
Ara C 100 mg/m^2 days 1, 5 DNR 60 mg/m^2 days 1, 2	31	15 (48%)	2	3	11
Asparaginase 1000 U/kg/day days, 1, 3 Cycl. 400 mg/m^2 day 5 Ara C 100 mg/m^2 days 5, 10 DNR 60 mg/m^2 days 5, 6, 7	43	21 (48%)	2	10	10
Total	159	77 (48%)	8	30	44

[a] Drug abbreviations: DNR: daunorubicin; Ara C: cytosine arabinoside; Cycl.: cyclophosphamide.

normal donors (37, 38); our experience is based upon 66 centrifugations in the NCI-IBM continuous flow blood cell separator utilizing normal donors' blood in 41 leukemic patients with severe infections. Here, compatibility in the ABO Rh system is imperative: tests of lymphocytotoxicity and leucoagglutination between the recipients' serum and the donors' leucocytes must be negative. Prior administration of hydrocortisone to the donor and addition of modified liquid gelatine (plasmagel) to the separated blood improve the recovery of granulocytes, which is about 50 X 10^9 per centrifugation. Transient responses are obtained in 44% of patients and definite improvements are noted in 35% of cases.

Duration of Complete Remissions and Survival

Until recently, attempts to maintain a remission in acute myeloid leukemia have been relatively ineffective. Maintenance with 6-mercaptopurine (6-MP) and intermittent methotrexate (MTX), with reinductions with DNR and Methyl-GAG, gave a median remission length of 7 months. Gluckman et al (29), after induction with Ara C and DNR, report the same median length of 12 months in 11 patients given bimonthly Ara C-DNR and 4 patients given 6-MP and Methyl-GAG and reinductions with DNR and Ara C. Whitecar et al (31) report a median length of 12 months using bimonthly courses of combination therapy (COAP) in 17 patients. However, the most interesting results are those of Powles et al (39) who compared 23 patients in CR randomly allocated to receive alternating monthly courses of Ara C and DNR or Ara C and TG, plus weekly immunotherapy (allogenic irradiated leukemic cells and BCG), to 18 patients allocated to the same chemotherapy without immunotherapy. The median length of CR has been 12 months in the first group and 6 months in the second. The possible role of the stimulation of the patients' response to blastic cells emphasizes the importance of the information reported by Hersh et al (40). Of 18 patients showing delayed hypersensitivity after immunization, 13 achieved CR, whereas among 7 patients without this reactivity, only 2 entered CR. Conversion from immunoincompetence to immunocompetence during treatment also carries a good prognosis. Survival in AML depends upon whether the patient attains remission or not; in the nonresponder survival is disturbingly short (less than one month), and this may be ascribed to the natural course of the disease as well as to drug toxicity. Even in the responder the median survival does not exceed 12 months, although an increased percentage of patients live for more than two years and long term survivals may be observed in AML as in ALL. Of the 23 four-year survivors of acute granulocytic leukemia who were treated in our department, six have died between four and seven years after diagnosis; 13 patients are still in CR (four had acute promyelocytic leukemia) and three are still alive after a relapse.

SPECIFIC THERAPEUTIC PROBLEMS OF ACUTE PROMYELOCYTIC AND ACUTE MONOBLASTIC LEUKEMIAS

Promyelocytic leukemias were recorded before 1967 as the most severe variety of AGL (41–43), with a three-week median survival and hemorrhagic deaths. Since DNR has been available, the rate of CR has risen from 13% of 36 patients to 58%

of 44 patients; whereas the hemorrhagic risk is high during the five days after diagnosis, it becomes negligible thereafter once medullary aplasia is obtained (43, 44).

Hemorrhage in most cases is due to disseminated intravascular coagulation (DIC) as assessed by fibrinogenopenia and the existence of fibrinogen-reactive antigens (FRA) and soluble complexes (SC). DIC, induced by the thromboplastic activity of the malignant promyelocytes (45), may be initiated by the cellular lysis as produced by the first doses, particularly in patients with more than 1000 leucocytes/mm^3. Thus, DNR should be regarded as an emergency treatment in APL with the same schedule and doses as in AML. In the high risk patients, those with an initial fibrinogen level of less than 1 g/100 ml or with a relatively high peripheral promyelocyte count, heparin (1 mg/kg/day) and platelet transfusions (6 U/m^2) are indicated until aplasia is obtained. With a maintenance treatment of 6-MP and MTX and with or without reinductions with DNR and Methyl-GAG, the duration of the complete remissions is remarkably long, a median of 26 months as compared to the 7-month median in AML. The expectancy of an unusual number of long term survivals is thus now probably the most interesting feature of acute promyelocytic leukemia.

Like APL, acute monoblastic leukemias were considered "very acute leukemias" (43). Diagnosis of acute monoblastic leukemia relies on cytological (46), and above all on cytochemical characteristics (47–49): the naphthol AS acetate esterase activity of monoblasts is inhibited by sodium fluoride. These cells present particular biophysical features: adhesiveness, cell electrophoretic mobility, cellular deformability, and phagocytosis, which may explain the particular hematological and clinical picture in this leukemia (50). High leucocyte count and major tissue involvement such as skin infiltration and hypertrophic gingivitis are more frequently found in acute monoblastic leukemias than in other varieties of AGL. Severe hyperuricemia is often observed before any therapy and the treatment after 36 hours of allopurinol administration and hyperdiuresis should be progressive in order to avoid impairing renal function. We achieved nine complete remissions in ten children and two in eleven adults. Relapses were observed in one adult after one month and in four children after two, five, six, and eight months, respectively. Skin infiltration (nine cases) does not appear to worsen the prognosis, at least in children (five complete remissions). Two patients had meningeal leukemia during hematological remission. Therefore, we think that in childhood acute monoblastic leukemia there is an indication for prophylactic skull irradiation and intrathecal methotrexate doses once CR is attained.

LEUKEMIA WITH AN EXCESS OF UNDIFFERENTIATED CELLS AND ABNORMAL GRANULOCYTIC PROLIFERATION

This group includes several syndromes which, in addition to clinical and hematological ambiguity, have raised much semantic confusion. Their nature is not yet clarified but they should not be confused with acute myeloblastic leukemia since their therapeutic implications are different. Although they may terminate in infection or hemochromatosis, they share the common future of ending possibly after some months or years as severe and therapeutically resistant acute myeloblastic leukemia.

Erythroleukemias exhibit high counts of circulating erythroblasts ($\geq$ 50%) as well as undifferentiated cells, have the same sensitivity to chemotherapy as typical forms of acute myeloblastic leukemia, and should be treated in the same fashion.

Subacute leukemias are a heterogeneous group of diseases with an excess of blastic cells and increased activity of the proliferative granulocytic pool. It would be useful to classify these leukemias according to clinical, cytological, and cytogenetical studies (51); we hope that technics such as semisolid or liquid cultures of bone marrow will supply further criteria and confirm or deny the value of this tentative classification (52).

Subacute leukemias with the Ph_1 chromosome differ from chronic myelocytic leukemia because of poor patient condition with fever and bone pains and an excess of blastic cells. Prognosis seems better in those forms in which there is bone marrow myelofibrosis than in the forms in which the blastic infiltration predominates and splenomegaly is considerable.

In subacute leukemias without the Ph_1 chromosome two varieties may be distinguished. Myelomonocytic subacute leukemias can be recognized by the coexistence of immature granulocytes, mature monocytes, and high lysozyme levels both in blood and urine. In the other variety, qualitative defects are present in eythrocytes and platelets and there is a marked increase of polyclonal immunoglobulins (53).

Acute myelofibrosis is distinct from acute myeloblastic leukemia with myelofibrosis only by the absence of organ involvement and splenic metaplasia (54).

All these conditions are generally impaired by aggressive treatments that aim toward aplasia, but some improvement may be obtained with the combination of hydroxyurea, 6-MP, and prednisone in small dosage.

Refractory anemias with an excess of blastic cells are described in adults (55). In other patients sideroblastic anemia with qualitative defects in the other series, or refractory neutropenia or refractory thrombocytopenia, may precede an overt acute leukemia by several months (56). Although it is infrequent, such conditions may be observed in children. These syndromes share the common feature of being most often resistant to chemotherapy: aplasias are obtained but repopulation is abnormal.

We shall not discuss here whether those cases illustrate the "one population" theory (32) or merely the fact that more mature cells are less sensitive to chemotherapy. In answer to the question raised by Crosby (57) in 1968, "to treat or not to treat" acute granulocytic leukemias, a positive response can now be given. Like Crosby, we think that the recognition of different varieties of AGL is important. Among refractory varieties of AGL this author included promyelocytic leukemia, AGL in the elderly, smouldering leukemias, AGL with megaloblastosis, and blastic crisis.

As we have already stated, promyelocytic leukemias are the most sensitive form of AGL, and their treatment should be considered as an emergency.

It is hoped that the discovery of new drugs or new methods of treatment will improve the prognosis in all patients and will further reduce the number of resistant forms of AGL.

Literature Cited

1. Witts, L. J. et al 1966. *Brit. Med. J.* 1: 1383
2. Frei, E. III et al 1961. *Blood* 18:431–54
3. Levin, R. H., Henderson, E., Karon, M., Freireich, E. J. 1965. *Clin. Pharmacol. Ther.* 6:31–42
4. Ellison, R. R. et al 1968. *Blood* 32: 507–23
5. Bernard, J., Boiron, M., Jacquillat, Cl., Weil, M., Najean, Y. 1966. *Presse Med.* 74:700–802
6. Boiron, M. et al 1969. *Lancet* 1:330–33
7. Weil, M. et al 1973. *Cancer Res.* 33: 921–28
8. Wiernik, P. H., Serpick, A. A. 1972. *Cancer Res.* 32:2023–26
9. Jacquillat, Cl. et al 1972. *Brit. Med. J.* 941:468–70
10. Cortes, E. P., Ellison, R. R., Yates, J. W. 1972. *Cancer Chemother. Rep.* 56(2): 237–45
11. Chu, M. Y., Fischer, G. A. 1962. *Biochem. Pharmacol.* 11:333–41
12. Schrecker, A. W., Urshel, M. J. 1967. *Proc. Am. Assoc. Cancer Res.* 8(230):58
13. Camiener, G. W., Smith, C. G. 1965. *Biochem. Pharmacol.* 14:1405–16
14. Kessel, D., Hall, T. C., Rosenthal, D. 1969. *Cancer Res.* 29:459–63
15. Steuart, C. D., Burke, P. J. 1971. *Nature New Biol.* 233:109–10
16. Bernard, J., Jacquillat, Cl., Weil, M. 1972. *Semin. Hematol.* 2:181–91
17. Greene, W. et al 1972. *Cancer* 30: 1419–27
18. Huffman, D. H., Bachur, H. R. 1972. *Cancer Res.* 32:600–5
19. Macrez, C. et al 1967. *Pathol. Biol.* 15: 949–53
20. Malpas, J. S., Scott, R. B. 1968. *Brit. Med. J.* 3:227–29
21. Bonadonna, G., Monfardini, S. 1969. *Lancet* 1:837
22. Bonadonna, G. et al 1969. *Brit. Med. J.* 3:503–6
23. Mathé, G. et al 1970. *Presse Med.* 78: 1997–99
24. Freireich, E. J. et al 1970. In *Recent Results in Cancer Research,* 30:85–91. New York: Springer Verlag
25. Gee, T. S., Yu, K. P., Clarkson, B. D. 1969. *Cancer* 23:1019–32
26. Carey, R. W. 1970. *Proc. Am. Assoc. Cancer Res.* 11:55
27. Dowling, M. G., Gee, T. S., Lee, B. J., Clarkson, B. D., Burchenal, J. H. 1972. *Proc. Am. Assoc. Cancer Res.* 13:21
28. Crowther, D., Powles, R. L., Bateman, C. J. T. 1973. *Brit. Med. J.* 1:131–37
29. Gluckman, E., Basch, A., Varet, B., Dreyfus, B. 1973. *Cancer* 31:487–91
30. Rosenthal, D. S., Moloney, W. C. 1972. *N. Engl. J. Med.* 286:1176–78
31. Whitecar, J. P., Bodey, G. P., Freireich, E. J., McCredie, K. B., Hart, J. S. 1972. *Cancer Chemother. Rep.* 56:543–50
32. Clarkson, B. D. 1972. *Cancer* 30: 1572–82
33. Bodey, G. P., Gehan, E. A., Freireich, E. J., Frei, E. III. 1971. *Am. J. Med. Sci.* 262:139–51
34. Freireich, E. J., Judson, G., Levin, R. H. 1965. *Cancer Res.* 25:1516–20
35. Bussel, A., Benbunan, M., Tanzer, J., Boiron, M. 1971. *Actual. Hematol.* 5: 169–80
36. Bussel, A., Benbunan, M., Tanzer, J., Bernard, J. 1973. *Transfusion de Leucocytes de Leucémie Myéloide Chronique.* Presented at IX Congrès Nat. Transfusion Sanguine, Nancy, May 1973
37. Graw, R. G., Herzig, G. P., Perry, S., Henderson, E. S. 1972. *N. Engl. J. Med.* 187:367–71
38. Benbunan, M., Bussel, A., Reviron, J., Bernard, J. 1973. *Experience Actuelle des Transfusions de Granulocytes Normaux Prélevé sur Séparateur de Cellules.* Presented at IX Congrès Nat. Transfusion Sanguine, Nancy, May 1973
39. Powles, R. L. et al 1973. *Immunotherapy in Acute Myeloblastic Leukemia.* Presented at 1st Meet. EORTC Tumours Immunology Group, London, 1973
40. Hersh, E. M., Whitecar, J. P., McCredie, K. B., Bodey, G. P., Freireich, E. J. 1971. *N. Engl. J. Med.* 22:1211–16
41. Bernard, J., Mathé, G., Coara, B., Chome, J. 1959. *Schweiz. Med. Wochenschr.* 89:604
42. Bernard, J., Boiron, M., Manus, A., Levy, J. P., Lellouche, J. 1964. *Nat. Cancer Inst. Monogr.* 15:359–66
43. Bernard, J., Boiron, M., Lortholary, P., Levy, J. P. 1965. *Cancer Res.* 25:457–59
44. Weil, M. et al 1972. *Actual Hematol.* 6: 4–13
45. Bernard, J. et al 1973. *Blood* 4:489–96
46. Quickley, J. H. 1967. *Fed. Proc.* 26: 648
47. Schumacher, H. R., Szekely, I. E., Park, S. A. 1973. *Cancer* 31:209–20
48. Brauwsteiner, H., Schmalz, L. F. 1968. *Nouv. Rev. Fr. Hematol.* 8:289–92
49. Daniel, M. T., Flandrin, G., Lejeune, F., Liso, P., Lortholary, P. 1971. *Nouv. Rev. Fr. Hematol.* 2:233–40

50. Flandrin, G., Daniel, M. T., Blanchet, P., Briere, J., Bernard, J. 1971. *Nouv. Rev. Fr. Hematol.* 11:241–54
51. Lichtman, M. A., Weed, R. I. 1972. *Blood* 40:52–61
52. Briere, J. et al 1973. *Rev. Prat.* 23:3957
53. Metcalf, D. et al 1972. *Proc. Int. Cancer Conf., Sydney, Australia, 1972,* 173–86
54. Briere, J. 1973. *Contribution à l'étude des leucémies myelomonocytaires subaigues de l'adulte.* Thesis, Univ. Paris, p. 127
55. Bergsman, K. L., Vanslyck, E. J. 1971. *Ann. Intern. Med.* 74:232–35
56. Dreyfus, B., Rochant, H., Sultan, C. 1969. *Nouv. Rev. Fr. Hematol.* 9:65–86
57. Crosby, W. H. 1968. *Arch. Intern. Med.* 122:79–80

Copyright 1974. All rights reserved

ANIMAL MODELS OF THE ASTHMATIC STATE[1]

❖7094

Roy Patterson, M.D. and Joseph F. Kelly, M.D.
Section of Allergy-Immunology, Department of Medicine, Northwestern University Medical School, Chicago, Illinois

INTRODUCTION

Asthma is a common but poorly understood response of the lower respiratory tract of humans, which has and will require considerable investigative effort in order to define the basic mechanisms involved. Only limited studies can be done in asthmatic humans for safety reasons. Appropriate animal models are necessary to test hypotheses of underlying mechanisms in this disease and to determine the significance of immunologic, pharmacologic, and cellular factors in human asthma. This discussion will include a brief review of the characteristics of asthma, the types of animal models that have evolved with comments on how these systems might relate to the human disease state, and, finally, summaries of some of the studies that have been carried out using these animal respiratory responses.

Human asthma has been characterized clinically as recurrent wheezing dyspnea occurring in patients with bronchial hyperreactivity to a variety of stimuli. Physiologically asthma is characterized by reversible obstruction of the bronchi, such reversibility demonstrated most commonly by the administration of β-adrenergic agonists. An additional characteristic has been the clinical experimental observation that the hyperreactivity of the bronchi can be demonstrated by the administration of histamine, methylcholine or, more recently, prostaglandin $F_2\alpha$ (1). In minimal asthma there may be no pathologic changes observed in respiratory tissues but in asthma of long duration a thickened basement membrane and eosinophilia have been found at autopsy (2). Immunologically, one type of asthma is related to reaginic antibodies of the E class of immunoglobulins. This type of asthma may be associated with elevated serum concentrations of IgE (3) and the specific IgE antibodies may be demonstrated by in vivo skin tests or in vitro radioimmunoassay systems.

Clinically, asthma is classified traditionally as either extrinsic or intrinsic. Extrinsic asthma is precipitated by inhalation of antigen, which reacts with IgE antibody fixed to mast cells in the lung with resultant mediator release. These mediators are

[1] Supported by USPHS Grants AI 11403 and AI 00057 and the Ernest S. Bazley Grant.

histamine, slow reacting substance of anaphylaxis (SRS-A), eosinophil chemotactic factor of anaphylaxis (ECF-A), and possibly others. Intrinsic asthma has no defined relation to IgE antibodies or to antibodies of other immunoglobulin classes and is a disease of unknown etiology. There are varying graduations between these two types of asthma and entities such as exercise-induced bronchospasm and the curious triad of aspirin allergy (which results in severe, perhaps fatal asthma), nasal polyposis, and asthma (4). Of these types of asthma, the only one simulated by current animal models is the extrinsic type associated with IgE antibodies, and it is in relation to this type of asthma that this review is primarily directed, since no animal models of intrinsic asthma have yet evolved. Three further characteristics of both extrinsic and intrinsic asthma are the association with eosinophilia in sputum and peripheral blood, hypersecretion of mucus, and the dramatic response to corticosteroids that almost always occurs in asthma and often persists at low maintenance doses.

There are three major current theories of the mechanism of asthma. The mediator release concept is based on evidence indicating that chemical agents are released by antigen-induced, IgE-mediated mechanisms. These agents include histamine, SRS-A, ECF-A, possibly prostaglandins, and others. The clinical picture of asthma is then produced by bronchoconstriction, bronchial edema, or both through the smooth-muscle-constricting and vasoactive properties of these agents. Such a mechanism is accepted as significant in reagin-mediated extrinsic asthma; there is little evidence that it is significant in intrinsic asthma.

The concept of an abnormality of the β-adrenergic mechanism in asthma is currently popular. Szentivanyi has formulated the hypothesis of a defective or blocked β-receptor mechanism (5) and cited various types of supportive evidence from animal experiments. The attractiveness and plausibility of this theory as an explanation for some or all cases of asthma has provided a stimulus to search for evidence in support of this concept in human asthmatics. Finally, the importance of the role of the parasympathetic nervous system as a major factor in the production of asthma is receiving increasing attention (6). The variety of nonimmunologic stimuli that produce changes in airway resistance in the asthmatic and experimental evidence of inhibition of bronchospastic activity by surgical or pharmacologic vagal block are providing increasing evidence for this concept. Neither of these latter two theories are incompatible with the IgE–mast-cell–mediator release phenomenon. Such an immunologic mechanism would be one type of stimulus capable of either initiating reflex mechanisms or of stimulating bronchial smooth muscle and mucosal inflammatory reactions that are not reversed by normal β-receptor activities. These theories of the pathogenesis of asthma are reviewed in more detail elsewhere (7). The relation of infection to intrinsic asthma and the response of asthma to corticosteroids are not explained by these theories.

ANIMAL MODELS WITH CERTAIN SIMILARITIES TO HUMAN ASTHMA

A disease characteristic of either the IgE-mediated (extrinsic) or non-IgE-mediated (intrinsic) type of asthma does not occur in animals with the possible exception of certain dogs, as described below.

Although asthma is said to occur in cats, there is no adequate medical documentation of this. A clinical syndrome of dyspnea in horses known as heaves (8) occurs and is related to exposure to moldy hay, but its similarity to any human disease is not established; it may be more closely related to the Farmer's Lung type of hypersensitivity pneumonitis than to asthma.

Guinea Pigs

The traditional immunologic model of antigen-induced airway obstruction has been the guinea pig. The actively immunized guinea pig challenged systemically with antigen develops anaphylaxis, the major manifestation and cause of death being acute airway obstruction. Animals may be immunized through the airway, challenged with antigen by that route, or both. Aerosol challenge with antigen results in the same acute respiratory obstruction (respiratory anaphylaxis) and this has been a standard model for antigen-induced airway response for many years (9). The limitation in size of the animals initially interfered with studies of pulmonary physiology but advances in technology have permitted sophisticated analysis of pulmonary mechanics in these animals (10). Guinea pig contractile tissues such as the tracheal ring (11) or the tracheal strip (12) continue to be of use in studies of smooth-muscle-constricting mediators released as a result of antigen-antibody reactions. In vivo and in vitro studies of guinea pig respiratory tissues are applicable to studies of reactivity of smooth muscle, cholinergic-adrenergic balance, and such materials as prostaglandin-like substances (13).

Dogs

With the exception of man, dogs are the only animals in which a defined hypersensitivity disease related to aeroallergens occurs (14). The clinical disease is most commonly ragweed pollenosis, although hypersensitivity to grass, tree, house dust, and cat antigens has been identified. The clinical manifestations include conjunctivitis, rhinitis, and an intensely pruritic dermatitis. In contrast to seasonal pollenosis in man, dermatitis, rather than ocular and respiratory symptoms, constitutes the major canine seasonal symptom. The mechanism of the dermatitis is not understood since the same mechanism that occurs in production of rhinitis and conjunctivitis (mucosal absorption of antigen reacting with sensitized mast cells) appears unlikely. The only immune response defined has been the presence of reaginic antibody; the percutaneous absorption of pollen protein antigens has not been demonstrated.

A clinical syndrome in dogs similar to asthma does occur in pollen-sensitive dogs manifested by cough, dyspnea, and production of thick ropey mucus. This is rare and although its incidence has not been adequately documented it appears to have a much lower rate of occurrence in allergic dogs than does asthma in allergic humans. In contrast to the rarity of lower respiratory tract response to environmental antigens, dogs with pollen allergy will almost always have a respiratory response to aerosolized antigen under controlled laboratory conditions as described below.

IMMUNOLOGY OF CANINE ALLERGY The primary clinical disease that has been characterized in dogs is ragweed pollenosis. These animals have a circulating antibody against ragweed antigen, which is heat labile, homocytotropic, of long latency, reaginic, and which appears to reside in a class of immunoglobulins analogous to human IgE (15). This reaginic antibody will passively transfer cutaneous reactivity, respiratory responsiveness, or anaphylactic sensitivity to normal dogs (14). Peripheral blood leukocytes of these dogs react with antigen to release histamine (16) in studies analogous to antigen-induced human peripheral blood histamine release (17).

ASCARIS REACTIVITY In studies parallel to those described below in rhesus monkeys, ascaris sensitivity in the dog has been evaluated (18). All dogs tested had cutaneous reactivity to a purified preparation of *Ascaris suum* antigen, presumptively due to cross reactivity of the ascaris antigen with antigens from *Toxacara canis.* Infestation with the latter nematode would be responsible for the primary immunologic sensitization of the dogs.

The dogs with cutaneous reactivity to ascaris antigen vary in the degree of reactivity as determined by cutaneous skin test titers (18). Many of these dogs have an immediate-type respiratory response to initial aerosol challenge. This system provides the most readily available model of acute antigen-induced airway response. Further studies have shown that dogs with cutaneous reactivity but no respiratory response to aerosol challenge with ascaris antigen will, after repeated aerosol exposure, develop respiratory responses. This is in contrast to such dogs aerosolized with ragweed antigen who do not develop respiratory reactivity to pollen antigen. Attempts to correlate the development of respiratory responsiveness to ascaris antigen with the presence of reaginic antibodies against ascaris antigen in serum and secretions have suggested that this correlation occurs (19).

The occurrence of canine ascaris respiratory reactivity makes dogs the most readily available large animals with reagin-mediated respiratory response available for study. The major limitations in the use of dogs for studies of acute, ascaris-induced respiratory responses is the variability of the response in the same animal even though the antigen dose is carefully controlled (19); this variability does not occur to the same extent in ascaris-reactive monkeys. A second limitation in the use of dogs is the "sudden death syndrome" (SDS) or apnea that occurs during aerosolized antigen administration in anesthetized dogs. SDS has occurred both in ascaris- and ragweed-sensitive dogs and is a very serious problem in the latter animals because of their limited availability. For this reason we do not use ragweed-sensitive dogs for controlled aerosolized antigen challenge under anesthesia. SDS has not occurred in unanesthetized dogs nor in either awake or anesthetized rhesus monkeys. The mechanism of the SDS is unknown but may be a central nervous system response producing apnea following reflex stimulation resulting from antigen-induced mediator release in the anesthetized animal.

Rhesus Monkeys

To our knowledge, IgE-mediated clinical disease has not been described in subhuman primates and we have not been able to identify pollenosis in these animals in limited evaluations at a local zoo. Such a disease state should occur, based on the studies to be reviewed below, and there is no good explanation why it has not been found.

The term homocytotropic has been applied to reaginic antibodies because these antibodies will usually sensitize skin of homologous recipients and not cross species barriers. An exception to this is the ability of human reagin to sensitize the skin of nonhuman primates. The transfer of cutaneous reactivity from humans to various monkeys, termed monkey passive cutaneous anaphylaxis (monkey PCA) is analogous to the human Prausnitz-Küstner reaction. The details of these early studies have been reviewed elsewhere (14).

Administration of sufficient amounts of human serum with IgE antibodies will result in systemic sensitization of monkeys and such monkeys will have anaphylaxis after intravenous injection of the appropriate antigen or an acute respiratory response if the antigen is delivered by aerosol. This passive respiratory response (PRR) is a reaction mediated by human IgE and as such constitutes an analogue of IgE-mediated reactions in man although the degree of similarity to human IgE-mediated asthma is uncertain. There is good evidence that such respiratory reactions in monkeys are dependent upon the pathway of mast cells and basophils sensitized by IgE releasing vasoactive and smooth-muscle-constricting agents following exposure to antigen. If human asthma of the IgE-mediated type requires that the asthmatic individual have an additional nonimmunologic, physiologic abnormality such as either a defective β-adrenergic receptor mechanism, a heightened parasympathetic responsiveness, or both, the passively sensitized rhesus monkey would then constitute a model of only one of the potential triggering mechanisms.

A major limitation in the use of the monkey passively sensitized by human IgE antibodies is that these animals require resensitization for each experiment. The IgE antibody is neutralized following exposure to sufficient antigen and the reaction will be negative or markedly reduced following a second exposure to antigen. Although the animal may be resensitized with repeat injections of human IgE, this is followed by a refractory state presumptively due to the rhesus monkey making antibody against the heterologous IgE (14).

ASCARIS SENSITIVITY IN RHESUS MONKEYS Other systems for study of reagin-mediated reactions in rhesus monkeys have been evaluated. We have found that certain rhesus monkeys have a significant degree of immediate-type hypersensitivity to *Ascaris suum* antigen, presumably due to prior sensitization by nematode infestations. The rhesus reaginic antibody mediating this reactivity (20) has the characteristics of human reagin and is probably rhesus IgE. Selected animals have a marked degree of cutaneous reactivity to ascaris antigen as shown by skin dilution titration, and certain of these animals will have immediate-type respiratory responses to aerosolized antigen. The respiratory responsiveness of these animals to antigen challenge may be present for long periods of time, up to three years in one group of animals we have studied (21). Although the degree of respiratory response to antigen is somewhat variable on each occasion, the responses are sufficiently reproducible to provide a pharmacologic test system for evaluation of the action of such agents as antihistamines, disodium cromoglycate, and β-agonists. The major limitation in this system is that the animals with respiratory reactivity to ascaris antigen constitute a small portion of unselected animals, which is estimated at less than 5%. In spite of the latter objection, the ascaris-antigen-induced respiratory response probably constitutes the best in vivo test system for reagin-mediated respiratory responses in terms of availability, reproducibility, and duration of responsiveness.

REVERSE PASSIVE RESPIRATORY RESPONSE It was recognized that antihuman IgE specific for the Fc fragment of IgE would cross react sufficiently with determinants in the heavy chains of monkey IgE to produce reverse passive cutaneous reactions in monkey skin (22). The mechanism for such reactions is considered to be the bridging of IgE molecules on the surface of the mast cell by the anti-IgE in a manner somewhat analogous to the bridging of IgE antibodies by antigen (23). Evidence of the bridging of the anti-IgE was based on the activity of whole anti-IgE or $F(ab')_2$ fragment against IgE in the release of mediators from human basophils and monkey lung tissue (22). These bivalent molecules produce the reaction while F(ab') fragments of anti-IgE do not, presumably because the latter are monovalent and do not bridge the IgE molecules on the surface of reactive cells (22).

On the basis of the above, we proposed that antihuman IgE that cross reacts with rhesus IgE should produce immediate-type respiratory responses in monkeys following aerosol delivery of anti-IgE, or anaphylaxis following intravenous injection. Anti-IgE does produce an acute respiratory response characteristic of the antigen-induced respiratory responses (24). These responses could be either potentiated or inhibited by prior administration of human IgE to the monkey, and which occurred appeared to be dependent on the amount of IgE and the time of administration in relation to challenge (25). Further, as predicted, intravenous administration of antihuman IgE to rhesus monkeys results in anaphylaxis (26), characteristic of the result of antigen administration to monkeys that have been passively sensitized with human IgE antibodies (14). The reverse passive systemic anaphylaxis due to anti-IgE provides a readily available model of primate anaphylaxis for study of systemic and respiratory physiologic abnormalities during anaphylaxis and provides in vivo release of mediators including histamine and possibly others (26).

The reverse passive respiratory response (RPRR) due to anti-IgE was initially considered to be the most potentially useful for studying immediate-type respiratory reactions in primates because all normal animals have IgE as demonstrated by the reverse passive cutaneous anaphylaxis reaction. Following detailed analysis of the RPRR, it was found that reproducible responses occurred in some animals, and that many animals reacted to aerosolized anti-IgE. However, there was not the complete consistency in response in individual animals or in groups to provide a superior model to the ascaris-sensitive animal.

The types of respiratory responses described above and their immunologic mechanisms and limitations are described in Table 1.

PHYSIOLOGY

The physiologic changes observed in human bronchial asthma include reversible airway obstruction, nonuniformity of ventilation, regional change in ventilation/

Table 1 Summary of some characteristics of animal models of reagin-mediated respiratory responses

Type of Reactivity	Antigens Used	Sensitization	Availability	Immunology	Respiratory Response	Produced in Normal Animals	Limitations
Dogs							
Active pollen sensitivity	Ragweed and grass; Others feasible	Result of environmental exposure	Not rare but very difficult to obtain for study	Reaginic antibody; Probably IgE	Positive on repeated exposure; Duration for many years	No	Rarely available. "Sudden death syndrome"
Passive sensitization	Ragweed; Others feasible	Passive sensitization with canine reagin	Requires serum donors	Canine reaginic antibody	Positive if sensitizing dose sufficient (volume and titer)	Yes	Requires repeated sensitization for each experiment
Active ascaris sensitivity	Purified *Ascaris suum* antigen	Result of infestation with *T. canis*	Readily available	Reaginic antibody; Probably IgE	Positive but varies in intensity	Occurs in most dogs after aerosol exposure	Variability of respiratory responses
Rhesus Monkeys							
Active ascaris sensitivity	Purified *Ascaris suum* antigen	Result of previous nematode infestation	Moderately difficult to obtain	Reaginic antibody; Probably IgE	May persist for years or decline and disappear	Cannot be induced by exposure	Not readily available
Passive sensitization	Varies; Ragweed and others	Sensitization with human IgE antibodies	Readily available	Human IgE antibodies	Positive if serum reagin titer and dose sufficient	Yes	Requires repeat sensitization. Animals become refractory.
Reverse passive respiratory response	IgE on surface of mast cell or basophil	Animal's own IgE	Only certain animals reactive to aerosol	Rabbit antihuman IgE cross reacts with monkey IgE on mast cells	All animals do not react. Some animals are repeatedly positive	Yes	All animals do not react

perfusion ratio, arterial hypoxemia, and frequency-dependent compliance changes (27). Animal models of asthma, characterized by the dog and monkey systems, have demonstrated many of these features. The use of general anesthesia in the experimental models may have altered some physiologic responses and all comparisons with the human disease in the unanesthetized state should be considered in this context.

Rhesus Monkey Systems

The initial evaluation of respiratory responses was performed in unanesthetized, passively sensitized rhesus monkeys exposed to ragweed antigen. Clinical observation disclosed rapid, shallow respirations, a prolonged expiratory phase (subsequently measured as a change in the expiratory time/inspiratory time ratio or *E/I* ratio), occasional gasping respirations, and very rarely wheezing, which was heard on auscultation (28). These changes were acute in onset, occurred only after exposure to appropriate antigens, and reverted to normal with time or more rapidly with epinephrine treatment. These findings were considered to resemble the acute asthmatic episode in humans, at least in certain aspects.

Subsequent evaluation was performed under general anesthesia with a cuffed endotracheal tube used to obtain an air flow tracing (29, 30). The physiologic parameters that were measured included frequency of respirations (*f*), *E/I* ratio, and peak expiratory flow rate (*PEFR*). Under these conditions, an increase in *f* and *E/I* ratio and decrease in *PEFR* was associated with the inhalation of histamine. When similar changes were induced in the active respiratory response (ARR), PRR, and RPRR, criteria for a positive response were suggested (31). A typical example of a positive response by these criteria is shown in Figure 1. These changes correspond well with the clinical observations of human disease and the simple parameter (*PEFR*) frequently used to assess asthmatic patients. These typical changes were found to be reproducible in selected animals and prompted the use of these immediate-type, immunologically mediated respiratory responses to study agents that enhance or inhibit responses (31–33).

The clinical response in unanesthetized animals, the reproducibility and character of the responses (notably the increased *E/I* ratio and decreased *PEFR*) to immunologic stimuli, and the similarity of the histamine response to the immunologic response all strongly suggested that these typical changes were representative of reversible obstructive airway disease. More detailed evaluation of the responses to histamine, ascaris antigen, and anti-IgE have recently been completed (34, 35). These studies indicate that the typical changes of increased *f* and *E/I* ratio and decreased *PEFR* in response to histamine, ascaris antigen, or anti-IgE are associated with a definitive increase in pulmonary resistance (*PR*) which parallels the other physiologic abnormalities. These findings confirm that the ARR and RPRR are a model of acute reversible airway disease, similar to human extrinsic asthma. Certain animals that have marked cutaneous sensitivity to ascaris antigen and that do not develop the typical *f*, *E/I* ratio, and *PEFR* changes may demonstrate increased *PR* on ascaris challenge indicating that *PR* is a more sensitive parameter of response in these animals.

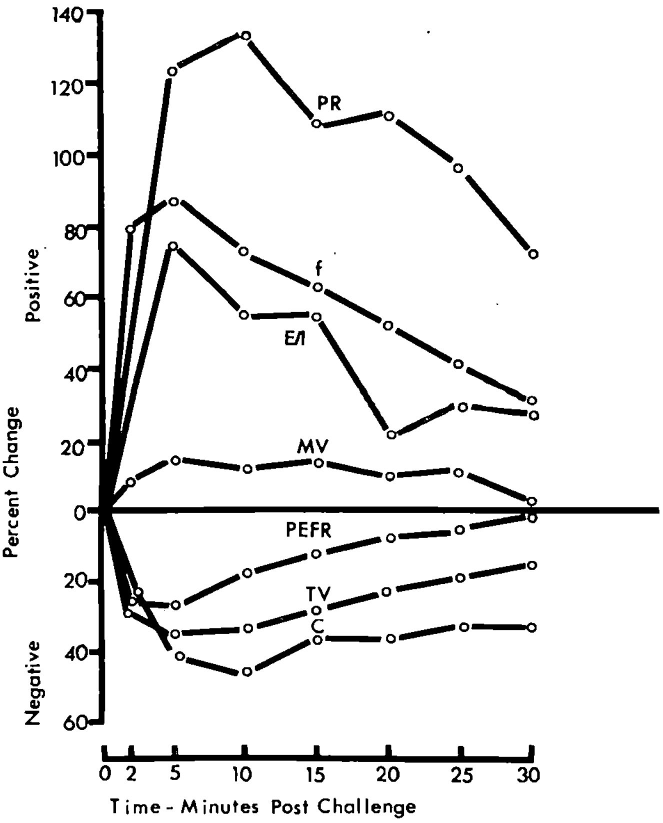


Figure 1 The percent change from control values after challenge in the respiratory parameters of an anesthetized rhesus monkey exposed to ascaris antigen aerosol. Abbreviations: *PR*, pulmonary resistance; *f*, breathing frequency; *E/I*, expiratory-inspiratory time ratio; *MV*, minute volume, *PEFR*, peak expiratory flow rate; *TV*, tidal volume; *C*, dynamic compliance.

One additional change in pulmonary function that has been observed in the respiratory response of the rhesus monkey is of interest. The dynamic compliance (*C*) decreases markedly in response to immunologic stimuli or histamine. Such a change may be related to hyperinflation as has been suggested by clinical observation in these animals. In addition, a reciprocal correlation exists between the increased *f* and the decreased *C* and thus suggests. the *f*-dependent *C* changes of human asthma (36, 37). This finding is under further evaluation at the present time, but may be representative of the nonuniformity of ventilation noted in asthma.

The pulmonary function parameters that can be determined in animals are limited to those that are effort-independent. Commonly used parameters of pulmonary function in human asthma, such as the timed vital capacity, maximal breathing capacity, and maximal expiratory flow rate, are all effort-dependent and hence are unsuitable for animal studies. Human studies suggest the *PR* may be abnormal in asymptomatic asthmatics (27) and that frequency-dependent compliance change may even be a man-sensitive parameter in the asthmatic (37). The findings in the monkey system tend to correlate well with human studies in that *PR* and reciprocal *f-C* changes are more sensitive than the *f*, *E/I* ratio, and *PEFR* changes originally reported.

The pathophysiologic response of human asthma is presumed to include edema of the bronchial mucosa, constriction of bronchial smooth muscle, and the secretion and accumulation of viscous mucus in many patients. The end result of these events is to impede airflow in the respiratory tract. In the monkey, changes in *PEFR, E/I* ratio, and *PR* probably reflect these abnormalities in the respiratory tract. The reciprocal *f-C* changes may reflect similar events in peripheral airways.

Blood gas analyses have been performed in the rhesus monkey during passive respiratory reactions (38). The responses were associated with significant decreases in pO_2, variable increases in pCO_2, and slight decreases in pH. The variable changes reported are suggestive of similar values reported in humans during acute asthma (39) with the most frequent change being hypoxemia.

Canine Systems

Canine respiratory responses have been induced by immunologic stimuli in both the anesthetized and unanesthetized animal. In many respects, the changes in lung function resemble parallel experiments reported previously in the rhesus monkey.

The changes that occur in the unanesthetized dog may be observed by clinical evaluation or permanent records obtained by the recording of a pneumograph tracing. When the allergic animal is exposed to the appropriate aerosolized antigen in a chamber, acute dyspnea will develop. That is, increased frequency of respirations and labored breathing will occur (14, 40). If the animal is removed from the test chamber, gradual recovery occurs, and if the dog is treated with epinephrine, there is more rapid resolution of these respiratory changes. Physical examination of these animals has not revealed wheezing.

By using an endotracheal tube in anesthetized dogs and recording airflow after the controlled administration of an antigen aerosol by a respirator, the *f*, *E/I* ratio, tidal volume (*TV*), and *PEFR* have been studied (14). The observed changes resemble those obtained in the monkey and include increased *f* and *E/I* ratio and decreased *TV* and *PEFR*. In more recent studies, we (19) and others (41–43) have demonstrated an increase in *PR* in response to antigen challenge in ascaris-sensitive dogs. These changes are reversed by epinephrine. The changes in canine respiratory function described above have been demonstrated in both the ARR and PRR.

Certain studies have suggested that respiratory immunologic response to ascaris antigen in both the anesthetized and unanesthetized dog is due to reflex activity mediated by the vagus nerve (41, 43). The increase in *PR* induced by ascaris antigen in sensitive animals may be blocked by pretreatment with atropine, reversed by the subsequent administration of atropine, and inhibited by sectioning or by cooling the vagus nerve (41). Further studies supporting the importance of reflex activity in respiratory responses to ascaris were done using a double-lumen endotracheal tube (41). Using this method, the right and left main stem bronchi were isolated and permitted the administration of antigen and the subsequent measurement of *PR* in the right or left lung. Antigenic stimulation of one lung resulted in increased ***PR*** in both lungs, an effect that was inhibited by cooling the vagus nerve innervating the challenged lung. In addition, electrical stimulation of the vagus nerve without antigen challenge results in measureable increases in airway resistance, either in central airways, peripheral airways, or both (44). Such studies support the importance of the role of reflex mechanisms in canine respiratory reactions induced by ascaris. Similar studies have not yet been done in the monkey.

Table 2 summarizes some of the respiratory function abnormalities found in human asthma with the changes in respiratory function found in the animal models on exposure to the appropriate stimulus.

Table 2 Comparison of changes in pulmonary function parameters of suitably sensitized humans, dogs, and rhesus monkeys when exposed to the appropriate antigen aerosol

Parameter	Human	Monkey	Dog
Frequency of respirations	Increase[a]	Increase	Increase
Tidal volume	Decrease	Decrease	Decrease
Minute volume	Variable	Variable	Variable
Peak expiratory flow rate	Decrease	Decrease	Decrease
E/I ratio[b]	Increase[c]	Increase	Increase
Pulmonary resistance	Increase	Increase	Increase
Dynamic compliance	Decrease	Decrease	Decrease
f-C Change[d]	Present	Probable	Doubtful
Arterial pO_2	Decrease[e]	Decrease	Decrease
Arterial pCO_2	Increase[f]	Increase	Increase

[a] Indicates the usual direction of change in responses that are of sufficient intensity to affect the parameter under study.
[b] Expiratory time/inspiratory time ratio.
[c] Classical clinical observation is prolonged expiratory phase.
[d] Frequency-dependent compliance change.
[e] May be normal in mild responses.
[f] May initially decrease; increase indicates severe impairment of ventilation.

PHARMACOLOGICAL STUDIES

The development of the dog and monkey systems described above have allowed evaluation of certain pharmacologic agents that are of theoretical and practical interest in human asthma. Table 3 shows a partial list of agents that have been evaluated in these animal models. The monkey system may be more useful in drug studies affecting the antigen-induced respiratory response because the changes in the

Table 3 Partial list of pharmacologic agents evaluated in the canine and rhesus monkey respiratory responses to immunologic stimuli as compared to evaluation in human IgE-mediated asthma

Agent	IgE-Mediated Human Asthma	Monkey Respiratory Responses	Canine Respiratory Response	Comment[a]
1. Diphenhydramine	Partial inhibition*	Partial inhibition	-	1. May inhibit antigen-induced airway obstruction (AO) in some patients
2. Atropine	Insufficient data	-	Reverse*	2. Reverses antigen-induced AO
3. Isoproterenol	Inhibit or reverse	Inhibit or reverse	Reverse	5. Conflicting reports of clinical effectiveness (45–47)
4. Epinephrine	Reverse	Reverse	Reverse	6. Clinical effectiveness demonstrated in some patients
5. Diethylcarbamazine	Variable*	No effect	-	7. Acute airway obstruction caused by aerosol challenge
6. Disodium cromoglycate	Variable*	Partial inhibition	-	8. Acute airway obstruction
7. Histamine	AO*	AO*	AO*	9. Intravenous administration
8. Prostaglandin $F_2 \alpha$	AO*	AO*	-	10. No asthma in normals. May produce AO in some asthmatics (48)
9. SU 48/80	-	AO*	-	
10. Propranolol	Variable*	No effect*	-	
11. Norepinephrine	-	No effect*	-	10–13. Pretreatment did not affect pulmonary function parameters as response to antigen (preliminary data)
12. Propranolol + Norepinephrine	-	No effect*	-	
13. Phenylephrine	-	No effect*	-	

[a] Numbers in this column refer to agents listed in the first column. The comment refers to the species denoted by *.

respiratory parameters are more reproducible in separate experiments than in the canine system as used in our laboratory.

The animal systems may be useful in evaluating theories and mechanisms of human asthma by providing the in vivo correlate of various in vitro studies. The canine system has been used to support the concept that the autonomic nervous system may play a significant role in human asthma. In a similar manner, the theory postulated by Szentivanyi (5) that β-adrenergic blockade may play a role in human asthma should be able to be tested in animal models. β-Blocking agents and combinations of β-blocking agents and α-agonists did not enhance the respiratory response to ascaris in primates in our initial studies. This finding does not support Szentivanyi's theory, although appropriate pharmacologic blocking agents may not yet be available to provide the most suitable test system for evaluation of this hypothesis.

Evaluation of potential therapeutic agents for human asthma has been done to a limited degree (Table 3). Disodium cromoglycate caused partial inhibition of the respiratory response in ascaris-sensitive monkeys. In a similar study with diethylcarbamazine, no significant effect on the respiratory response was demonstrated. The study of drugs in these animal models may require a large number of experimental animals, especially if only partial inhibition of a response is obtained. These systems have the inherent variability of any biologic system. In addition, being whole animal systems, the variability may be enhanced by the level of anesthesia, endogenous hormone production, and fluctuations in the degree of immunologic reactivity.

IN VITRO CORRELATES OF IN VIVO IMMEDIATE-TYPE RESPIRATORY RESPONSES

The respiratory responses in intact animals are the result of highly complex interrelationships extending through reactions of antigen with IgE antibody, mediator release, action of mediators on receptor sites, vascular and smooth muscle response, and physiologic changes in the respiratory tract that are modulated by reflex mechanisms. Dissection of the individual steps in a system of this complexity cannot be worked out in the whole animal and requires in vitro systems for analysis of the characteristics and the factors that affect the individual processes involved.

The use of peripheral blood leukocytes from human subjects with IgE-mediated respiratory disease has provided a model system for in vitro study of antigen-induced mediator release. This model has been reviewed elsewhere in detail (49). Using this system, studies have progressed from evaluation of mediator release to the modulation of antigen-induced mediator release by a variety of substances, of which agents that increase cyclic AMP are of particular interest (50).

Another related model of similar importance is the IgE-mediated release of vasoactive and smooth-muscle-stimulating agents from primate lung. A review of the biochemical and other factors involved in immunologic release of mediators of immediate-type hypersensitivity has been written by Orange & Austen (51) and is of particular value in relation to SRS-A. The human lung system has also been of considerable value in studying the release of histamine, SRS-A, and ECF-A. The release of these agents is affected by β-adrenergic and prostaglandin stimulation, which produces suppression, and α-adrenergic and cholinergic stimulation, which produces enhancement (52).

Rhesus and Canine Mast Cell Systems

The release of mediators from human lung treated as described above generally requires tissue from unselected subjects undergoing surgical procedures. We have evaluated the possibilities of obtaining antigen-reactive, mediator-releasing cells from pulmonary tissue without loss of the animal in the procedure. For this purpose the bronchial brush biopsy technique was used and the cells obtained from rhesus monkeys and human bronchi were studied (53 54). Mast cells are readily obtainable by this method, and these cells can be maintained in short-term tissue culture. The bronchial mast cells from rhesus monkeys with marked cutaneous and respiratory immediate-type hypersensitivity to ascaris antigen showed a significant degree of degranulation after exposure to antigen. Both human and rhesus monkey bronchial mast cells showed significant degranulation after exposure to rabbit antihuman IgE. The intracellular changes are observed directly as they occur in the cell. The cells are lightly stained with toluidine blue, which stains the granules without loss of viability or their ability to react with antigen or anti-IgE. When studied by dark field microscopy, the granules of these mast cells stained with toluidine blue appear yellow orange, which facilitates identification of the cells and permits observation of changes in the individual granules. Although this technique provides a source of viable mast cells from respiratory tissue, the major limitation in their use is that they are present in insufficient numbers from the bronchial brush biopsy samples to provide a source of cells for study of histamine and other mediator release.

More recently we have found that cells with metachromatic granules appear in the lumen of the canine and rhesus monkey respiratory tract. These cells constitute a small percentage (0.2–0.8%) of the total cell population (16, 55), but are consistently present in preparations of bronchial cells. Two types of cells with metachromatic granules are found: mast cells and cells that are the size and shape of basophils but more densely granular. The latter have been termed basophil-like until their identity is established by further study.

The studies conducted on these cells may be summarized as follows: A high percentage of the bronchial cells are viable. The histamine content is similar to that of the peripheral blood leukocytes of humans and dogs. They release histamine in response to specific antigen when they are obtained from an animal with IgE-mediated hypersensitivity (canine ragweed allergy), and this histamine release is paralleled by a degranulation process as described above. The source of these cells is uncertain but it appears that the basophils are not simply transferred from the peripheral blood because the cells have a different morphologic appearance than peripheral blood basophils, and the basophil-neutrophil ratio in bronchial cells is consistently much higher than that of peripheral blood.

These mast cells and basophil-like cells appear to be important because they are a source of respiratory antigen-reactive mediator releasing cells, but beyond this they may contribute significantly to immediate-type hypersensitivity responses of the lower respiratory tract. If the amounts of mediators released from these cells in the lumen are sufficient to cause vasoactive and smooth muscle response or to stimulate reflex bronchoconstriction they may be an important pathway in the production of asthma.

Literature Cited

1. Mathé, A. A., Hedqvist, P., Holmgren, A., Svanborg, N. 1973. *Brit. Med. J.* 1: 193–96
2. Aaronson, D. W. 1972. *Allergic Diseases: Diagnosis and Management,* ed. R. Patterson, 197–236. Philadelphia: Lippincott. 649 pp.
3. Johansson, S. G. O., Bennich, H., Berg, T., Högman, C. 1970. *Clin. Exp. Immunol.* 6:43–47
4. Samter, M., Beers, R. F. Jr. 1968. *Ann. Intern. Med.* 68:975–83
5. Szentivanyi, A. 1968. *J. Allergy* 42: 203–32
6. Simonsson, B. G., Jacobs, F. M., Nadel, J. A. 1967. *J. Clin. Invest.* 46:1812–16
7. Beall, G. N., Heiner, D. C., Tashkin, D. P., Whipp, R. J. 1973. *Ann. Intern. Med.* 78:405–19
8. Lowell, F. C. 1964. *J. Allergy* 36:322–30
9. Feinberg, S. M., Malkiel, S. 1952. *Proc. Soc. Exp. Biol. Med.* 81:104–5
10. Douglas, J. S., Lewis, A. J., Orehek, J., Bouhuys, A. 1973. *J. Clin. Invest.* 52:ix
11. Costello, J. C., deBeer, E. J. 1947. *J. Pharmacol. Exp. Ther.* 90:104–9
12. Patterson, R. 1958. *J. Allergy* 29:165
13. Drazen, J. M. et al 1973. *Fed. Proc.* 32: 1024
14. Patterson, R. 1969. *Progr. Allergy* XIII:332–407
15. Patterson, R., Roberts, M., Pruzansky, J. J. 1969. *J. Immunol.* 102:466–75
16. Patterson, R., Tomita, Y., Oh, S., Suszko, I., Pruzansky, J. J. *Trans. Assoc. Amer. Physicians* In press
17. Pruzansky, J. J., Patterson, R. 1966. *J. Allergy* 38:315–20
18. Booth, B., Patterson, R., Talbot, C. 1970. *J. Lab. Clin. Med.* 76:181–89
19. Patterson, R., Mellies, C. J., Kelly, J. F., Harris, K. E. In preparation
20. Weiszer, I., Patterson, R., Pruzansky, J. J. 1968. *J. Allergy* 41:14–22
21. Patterson, R., Talbot, C. 1972. *J. Allergy Clin. Immunol.* 49:292–300
22. Ishizaka, K., Ishizaka, T. 1969. *J. Immunol.* 103:588–95
23. Levine, B. B., Redmund, A. P. 1968. *J. Clin. Invest.* 47:556–67
24. Patterson, R., Talbot, C., Roberts, M. 1972. *J. Clin. Exp. Med.* 10:267–74
25. Kelly, J., Patterson, R. *Int. Arch. Allergy Appl. Immunol.* In press
26. Kelly, J. F., Patterson, R., Harris, K. E. In preparation
27. McFadden, E. R. Jr., Kiser, R., deGroot, W. J. 1973. *N. Engl. J. Med.* 288: 221–25
28. Patterson, R., Fink, J. N., Nishimura, E. T., Pruzansky, J. J. 1965. *J. Clin. Invest.* 44:140–48
29. Patterson, R., Miyamoto, T., Reynolds, L., Pruzansky, J. J. 1967. *Int. Arch. Allergy Appl. Immunol.* 32:31–45
30. Patterson, R., Talbot, C. 1969. *J. Lab. Clin. Med.* 73:924–33
31. Patterson, R., Talbot, C. 1972. *J. Allergy Clin. Immunol.* 49:292–300
32. Patterson, R., Talbot, C., Booth, B. 1970. *Am. Rev. Resp. Dis.* 102:412–21
33. Patterson, R., Talbot, C., Brandfonbrener, M. 1971. *Int. Arch. Allergy Appl. Immunol.* 41:592–603
34. Kelly, J. F., Cugell, D. W., Patterson, R., Harris, K. E. Submitted for publication
35. Kelly, J. F., Cugell, D. W., Patterson, R., Harris, K. E. Submitted for publication
36. Mills, R. J., Cumming, G., Harris, P. 1963. *J. Appl. Physiol.* 18:1061–64
37. Woolcock, A. J., Vincent, N. J., Macklem, P. T. 1969. *J. Clin. Invest.* 48:1097–1106
38. Miyamoto, T., Reynolds, L. B., Patterson, R., Cugell, D. W., Kettell, L. J. 1968. *Am. Rev. Resp. Dis.* 97:76–88
39. Bocles, J. S. 1970. *Med. Clin. N. Am.* 54:493–509
40. Patterson, R., Sparks, D. B. 1962. *J. Immunol.* 88:262–68
41. Gold, W. M., Kessler, G.-F., Yu, D. Y. C. 1972. *J. Appl. Physiol.* 33:719–25
42. Gold, W. M., Kessler, G.-F., Yu, D. Y. C., Frick, O. L. 1972. *J. Appl. Physiol.* 33:496–501
43. Dain, D., Hom, F., Gold, W. 1973. *Fed. Proc.* 32:415 (Abstr.)

44. Woolcock, A. J. et al 1969. *J. Appl. Physiol.* 26:806–13
45. Mallen, M. S. 1965. *Ann. Allergy* 23: 534–37
46. Benner, M., Lowell, F. C. 1970. *J. Allergy* 46:29–31
47. Srinivas, H. V., Antani, J. 1971. *Ann. Allergy* 29:418–21
48. Widdicombe, J. G., Sterling, G. M. 1970. *Arch. Intern. Med.* 126:311–29
49. Osler, A. G., Lichtenstein, L. M., Levy, D. A. 1968. *Advan. Immunol.* 8: 183–231
50. Lichtenstein, L., DeBernado, R. 1971. *J. Immunol.* 107:1131–36
51. Orange, R. P., Austen, K. F. 1971. *Progress in Immunology,* ed. B. Amos, 173–86. New York: Academic. 1554 pp.
52. Kaliner, M., Orange, R. P., Austen, K. F. 1972. *J. Exp. Med.* 136:556–67
53. Patterson, R., Suszko, I. 1971. *J. Immunol.* 106:1274–83
54. Patterson, R., Head, L., Suszko, I., Zeiss, R. 1972. *Science* 175:1012–14
55. Patterson, R., Tomita, Y., Oh, S., Suszko, I., Pruzansky, J. J. *Clin. Exp. Immunol.* In press

Copyright 1974. All rights reserved

SULFONYLUREAS AND THE HEART[1]

✧7095

Gerald S. Levey, M.D.,[2] *Kenneth C. Lasseter, M.D., and Roger F. Palmer, M.D.*
Division of Endocrinology and Metabolism, Departments of Medicine and Pharmacology, University of Miami School of Medicine, Miami, Florida

Sulfonylurea drugs such as tolbutamide have been used extensively in the treatment of patients with diabetes mellitus. The recent report of the University Group Diabetes Program (UGDP) (1) suggested an association of tolbutamide therapy with increased incidence of cardiovascular deaths. Little information is available concerning the direct cardiac effects of these agents in animals or man. This type of data becomes critical when attempting to understand possible mechanisms for the apparent increase in cardiovascular mortality in the UGDP study. For example, it has been shown that myocardial work and oxygen consumption are increased by agents which increase myocardial contractility such as calcium (2), catecholamines (3), and digitalis (4). Furthermore, inotropic agents administered to dogs prior to experimental coronary occlusion increase both the severity and extent of the myocardial injury (5). Similar effects can be postulated in a patient group (e.g. diabetics) having an increased incidence of coronary artery disease where there is a critical balance between myocardial oxygen supply and demand. It would not be unreasonable to consider such drugs potentially deleterious. In order to critically assess the sulfonylurea drugs in this light, we have attempted to review current information relative to the effects of these agents on various parameters of cardiac function in experimental animals and man.

ADENYLATE CYCLASE AND ADENOSINE 3', 5'-MONOPHOSPHATE

There are several mechanisms by which inotropic and chronotropic effects are produced. The cardiac effects of several hormones are thought to be mediated by

---

[1] Supported in part by USPHS Grant HE13710-3, by the Heart Association of Broward County, a chapter of the Florida Heart Association, and by Florida Heart Association Grant 71 RF 3.

[2] Investigator, Howard Hughes Medical Institute.

activation of the membrane-bound enzyme, adenylate cyclase, and the resultant increase in adenosine 3', 5'-monophosphate (cyclic AMP) (6, 7). Sutherland, Robison & Butcher have defined four criteria that should be fulfilled before the adenylate cyclase system can be considered to be responsible for mediating the physiologic effect of a hormone or drug on its target organ (6). First, the agent should have the capacity to increase the activity of adenylate cyclase in broken-cell preparations. Second, the level of cyclic AMP in whole-tissue preparations should increase in response to the hormone. Third, the physiologic response to the hormone should be potentiated by drugs such as theophylline that inhibit phosphodiesterase. Fourth, cyclic AMP or one of its derivations should mimic the physiologic effects of the hormone or drug. The sulfonylurea drugs have been examined in the context of these criteria. Thus far three of the four criteria have been fulfilled (8–10).

Tolbutamide activates myocardial adenylate cyclase over the concentration range 1 X 10^{-7} *M* to 1 X 10^{-5} *M* in the rabbit and 1 X 10^{-8} *M* to 1 X 10^{-6} *M* in humans (Figure 1). It would appear that the adenylate cyclase from human heart is more sensitive than that from the rabbit. However, both adenylate cyclases are activated by concentrations of tolbutamide well below those needed to obtain a therapeutic effect clinically (11), even considering the high affinity of these drugs for plasma proteins. In addition to the studies detailed in Figure 1, all of the sulfonylureas produced significant activation of rabbit myocardial adenylate cyclase (Table 1) compared to that produced by tolbutamide. These other sulfonylureas have not been tested in preparations of human heart. The sulfonylurea-mediated activation of

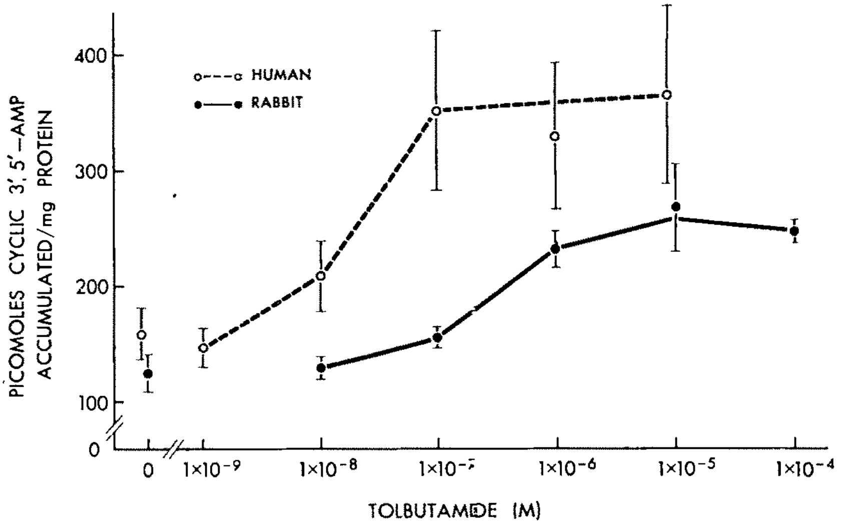


Figure 1 The effect of graded doses of tolbutamide in rabbit and human hearts. From Lasseter et al (9), with permission of the publisher.

Table 1 Effect of other hypoglycemic agents on rabbit and human myocardial adenylate cyclase[a]

Drug	Concentration (*M*)	Amount of cyclic AMP accumulated/mg protein/4 min[a] (pmol)
A. Control (rabbit)		190 ± 60
Tolbutamide	1×10^{4}	430 ± 60
Acetohexamide	1×10^{7}	270 ± 30
Acetohexamide	1×10^{6}	370 ± 3
Acetohexamide	1×10^{5}	440 ± 10
Acetohexamide	1×10^{4}	500 ± 40
B. Control (rabbit)		99 ± 22
Tolbutamide	1×10^{4}	165 ± 11
Chlorpropamide	1×10^{4}	165 ± 22
Tolazamide	1×10^{4}	209 ± 33

[a] From Lasseter et al (9) with permission of the publisher. Each value represents the mean ± SE of four samples.

myocardial adenylate cyclase is not blocked by the β-adrenergic blocking drug, DL-propranolol, indicating that this effect is not mediated by release of endogenous catecholamines. Finally, tolbutamide at 5×10^{-4} *M* increased the endogenous concentration of cyclic AMP (control 1.8 pmol/mg tissue to 4.4 pmol/mg tissue) simultaneously with the enhancement of contractility in isolated rabbit atria. Phenformin, a biguanide, did not activate adenylate cyclase in either rabbit or human hearts.

PHOSPHODIESTERASE

The other major intracellular regulator of cyclic AMP is phosphodiesterase, the enzyme which catalyzes the breakdown of cyclic AMP to the inactive 5'-AMP. In contrast to adenylate cyclase, which is located in the membranous structures of the cell, phosphodiesterase is mainly distributed in the soluble fractions of the cell. The relative activities of adenylate cyclase and phosphodiesterase determine the intracellular concentration of cyclic AMP. Phosphodiesterase is inhibited by the methylxanthines, theophylline and caffeine, a property which is utilized in in vitro assays of adenylate cyclase and which is thought to mediate many of the physiologic effects of these drugs. Recently, several studies have been reported demonstrating that tolbutamide also inhibits phosphodiesterase in a wide variety of tissues, including heart (12, 13), thereby providing another mechanism by which the sulfonylureas could increase intracellular cyclic AMP levels. Brooker & Fichman (12) found that chlorpropamide and tolbutamide inhibited rat kidney phosphodiesterase over the concentration range 5×10^{-4} *M* to 1×10^{-2} *M*. At peak concentrations there was approximately a 40% inhibition with either drug as compared to a 90% inhibition with equimolar concentrations of caffeine. Roth et al (13) confirmed these findings and showed that 5×10^{-3} *M* tolbutamide exhibited significant inhibition of phosphodiesterase in a variety of tissues in the rat including pancreas, brain, heart, kidney, liver, lung, and platelets. Brooker & Fichman have postulated that the inhibition of phosphodiesterase might decrease the degradation of cyclic AMP and result in higher intracellular levels of this cyclic nucleotide, thereby explaining some of the effects of sulfonylureas such as increased insulin release, antidiuresis in patients with diabetes insipidus, and the cardiovascular manifestations noted in the UGDP study.

INOTROPIC EFFECTS

Several investigations have established the positive inotropic effect of tolbutamide on rabbit (8, 9, 14), cat (15), and rat (16) heart muscle. Tolbutamide over the concentration range 1×10^{-5} *M* to 1×10^{-3} *M* produces a significant increase in contractility of isolated rabbit atria. The increase in contractility is seen after 1 min and is maximal within 10 min. Table 2 shows the effect of several sulfonylureas on contractility as compared to phenformin and norepinephrine. All the sulfonylureas tested exhibited a significant positive inotropic effect. Phenformin, a biguanide, produced no significant increase in contractile force and in actuality depressed contractility at the concentration tested. Results similar to those in the rabbit are seen in experiments utilizing isolated papillary muscles from cat heart (15).

As noted above, tolbutamide has been shown to inhibit phosphodiesterase in vitro. It has recently been reported that phosphodiesterase inhibitors such as theophylline potentiate the inotropic effects of glucagon (17) and norepinephrine (18), agents which are thought to exert their effects on the heart through the adenylate cyclase-cyclic AMP system. Lasseter et al found that the contractile response to tolbutamide is enhanced in the presence of theophylline; however, they could not demonstrate a true potentiation (9). In addition, pretreatment of isolated cat papillary muscles with tolbutamide, 2×10^{-4} *M*, failed to potentiate the concentration

Table 2 Effects of oral hypoglycemic agents on contractility of isolated rabbit atria[a]

Drug	Concentration (*M*)	% Control response[a]
Tolbutamide	5×10^4	124 ± 5
Acetohexamide	1×10^4	140 ± 5
Chlorpropamide	3×10^4	139 ± 6
Tolazamide	1×10^4	119 ± 6
Phenformin	1×10^3	85 ± 8

[a] From Lasseter et al (9), with permission of the publisher. The percent of control contractions after stabilization after drug addition expressed as mean ± SEM for 5–18 experiments with each drug.

response curve to norepinephrine (15). Since theophylline at equimolar concentrations effectively potentiates the contractile response to norepinephrine, these data suggest that tolbutamide is a less potent inhibitor of phosphodiesterase in vivo.

It also seems likely that the positive inotropic responses to sulfonylureas described above are species specific since no inotropic action has been observed secondary to the administration of tolbutamide to dogs (19, 20). The critical question pertaining to whether or not sulfonylureas have inotropic actions on human hearts remains unresolved. Epstein and co-workers (15) examined the effects of tolbutamide on two human papillary muscles obtained from patients at the time of mitral valve replacement for mitral stenosis and observed no inotropic effect. Crockett et al (21) studied the effects of acute oral or intravenous tolbutamide administration in four normal and eleven diabetic patients using as the index of response systolic time intervals, a noninvasive technique for measuring myocardial contractility. They could not demonstrate any positive effect of either oral or intravenous tolbutamide in either the normal or diabetic patients. In contrast to these studies, Hildner et al (22) studied 15 patients requiring cardiac catheterization for a variety of abnormalities excluding coronary artery disease. They found that a bolus injection of 250 mg tolbutamide intravenously produced a definite inotropic effect as reflected by ventricular function curves. In addition, they also demonstrated an inotropic effect of tolbutamide by noninvasive techniques using a carotid sensor to measure pre-ejection period and left ventricular ejection times. The pre-ejection period and left ventricular ejection times fell significantly within 15 min as did the ratio PEP/LVET. These alterations were maintained for about 30 min.

CHRONOTROPIC EFFECTS

Lasseter et al (9) showed that tolbutamide induced spontaneous activity in quiescent isolated dog Purkinje fibers. However, no alterations in heart rate in response to tolbutamide have been observed in rats (16), rabbits (9), or man (22).

CORONARY BLOOD FLOW

Khachadurian et al reported that tolbutamide decreased coronary blood flow in an isolated heart lung preparation in dogs (23). In experiments with open-chest and Starling dogs they found that coronary blood flow increased. Wittels & Hackel (24) found no effect of tolbutamide on coronary blood flow in dogs. There have been no reports on the effects of tolbutamide on coronary blood flow in humans.

CONCLUSIONS

The findings of the University Group Diabetes Program have posed a therapeutic dilemma for physicians concerned with the daily management of patients with diabetes mellitus. Studies of this nature are complex, require long periods of time, and are extremely difficult to reproduce. Not surprisingly the results have generated a good deal of controversy. Regardless of the outcome with respect to arguments concerning the adequacy of the design and interpretation of the results, the UGDP study has inspired a growing number of investigations pertaining to the effects of sulfonylureas on the heart. The information which has accrued from these studies and which will accrue from future studies is essential for any critical interpretation of the clinical findings of the UGDP study. It seems clear from several recent investigations that the sulfonylureas activate adenylate cyclase in the myocardium of rabbits and humans and exert positive inotropic effects in rabbits, cats, and rats. The inotropic effects in humans are less definite with two preliminary studies indicating no significant augmentation of contractility and one study demonstrating a significant inotropic effect. Several possible mechansims by which inotropic effects could prove deleterious and account for the increased cardiovascular mortality in the UGDP study have been discussed. Alternatively, it is also reasonable to speculate that the inotropic properties of these drugs might prove beneficial under certain circumstances of impaired cardiac function. Without doubt, many questions remain to be answered, both of a clinical and basic science nature, with regard to the therapeutic utility of sulfonylureas and in particular their role, if any, in increasing cardiovascular mortality in patients with diabetes mellitus. Although an intensive investigative effort will be required to resolve the controversy it is hoped that the necessary data will be forthcoming in the next few years.

Literature Cited

1. The University Group Diabetes Program. 1970. *Diabetes* 19: Suppl. 2, 747–830
2. Braunwald, E. 1971. *Am. J. Cardiol.* 27: 416–32
3. Graham, T. P. Jr., Covell, J. W., Sonnenblick, E. H., Ross, J. Jr., Braunwald, E. 1968. *J. Clin. Invest.* 47:375–85
4. Covell, J. W., Braunwald, E., Ross, J. Jr., Sonnenblick, E. H. 1966. *J. Clin. Invest.* 45:1535–42
5. Maroko, P. R. et al 1971. *Circulation* 43:67–82
6. Sutherland, E. W., Robison, G. A., Butcher, R. W. 1968. *Circulation* 37: 279–306
7. Epstein, S. E., Levey, G. S., Skelton, C. L. 1971. *Circulation* 37:437–50
8. Levey, G. S., Palmer, R. F., Lasseter, K. C., McCarthy, J. 1971. *J. Clin. Endocrinol. Metab.* 33:371–74
9. Lasseter, K. C., Levey, G. S., Palmer, R. F., McCarthy, J. 1972. *J. Clin. Invest.* 51:2429–34
10. Levey, G. S. 1972. *Controversies in Clinical Pharmacology and Drug Development,* ed. R. F. Palmer, 83–88. New York: Futura. 269 pp.
11. Sheldon, J., Anderson, J., Stoner, L. 1965. *Diabetes* 14:362–67
12. Brooker, G., Fichman, M. 1971. *Biochem. Biophys. Res. Commun.* 42: 824–28
13. Roth, J. et al 1971. *Ann. Intern. Med.* 25:607–21
14. Palmer, R. F., Lasseter, K. C., McCarthy, J. 1971. *Lancet* 1:604
15. Epstein, S. E., Grauer, L. E., Marcus, M. L. See Ref. 10, p. 99–107
16. Crass, M. F. III, Spanheimer, R. A., Stone, D. B., Brown, R. J. II. 1973. *Proc. Soc. Exp. Biol. Med.* 142:861–66
17. Marcus, M. L., Skelton, C. L., Prindle, K. H. Jr., Epstein, S. E. 1971. *J. Pharmacol. Exp. Ther.* 179:331–37
18. Skelton, C. L., Karch, F. E., Hougen, T. J. Marcus, M. L., Epstein, S. E. 1971. *J. Mol. Cell. Cardiol.* 3:243–53
19. deGuzman, N. T., Pinakatt, T., Yeh, B. K. 1972. *Proc. Fifth Congr. Pharmacol.,* p. 54
20. Mason, D. T., Zelis, R., Massumi, R. A., Amsterdam, E. A. See Ref. 10, p. 89–98
21. Crockett, S., Marsh, D., Lewis, R., Tzagournis, M. 1973. *Diabetes* 22: Suppl. I, 293
22. Hildner, F. J. See Ref. 10, p. 109–13
23. Khachadurian, A. K., Kapma, J. D., Badeer, H. S. 1961. *Arch. Int. Pharmacol. Dyn.* 132:42–48
24. Wittels, B., Hackel, D. B. 1961. *Proc. Soc. Exp. Biol. Med.* 107:375–77

Copyright 1974. All rights reserved

FOOD POISONING DUE TO *VIBRIO PARAHAEMOLYTICUS*

◆7096

William H. Barker, Jr., M.D. and Eugene J. Gangarosa, M.D.
Bacterial Diseases Branch, Bureau of Epidemiology, Center for Disease Control, Atlanta, Georgia

INTRODUCTION AND HISTORICAL ASPECTS

Vibrio parahaemolyticus is a neophyte among enteric pathogens. It was first recognized as a cause of food poisoning in Japan in the early 1950s (1), but its public health significance was not fully appreciated for a decade. Articles published in English do not make clear what factors contributed to its recognition in Japan. The importance of fish in the Japanese diet and the occurrence of large outbreaks along the Pacific Coast associated with marine foods (2) in the late 1950s undoubtedly played a significant role in stimulating microbiologists to search for organisms other than the known enteric pathogens, which were clearly not responsible for these outbreaks. The threat of invasion of El Tor cholera into Japan in the early 1960s also may have stimulated Japanese health authorities to search for vibrios in fecal cultures from persons with acute enteric disease. Finally, the introduction of new methods to culture vibrios also undoubtedly contributed. In any event it soon became evident that *V. parahaemolyticus* was the leading cause of foodborne illness in Japan, especially during the summer months, when it accounted for roughly 70% of all reported foodborne illnesses (3).

During the 1960s most public health workers considered this problem unique to Japan because of the apparent relationship with the popular Japanese custom of eating raw seafood. They were convinced otherwise a decade later because of events remarkably similar to those that had transpired earlier in Japan. In the early 1970s, El Tor cholera spread into the Middle East, Africa, and Spain and Portugal. Although there was no serious concern that cholera would spread in the United States, the introduction of disease via Americans returning from infected foci was considered a growing possibility (4). To ensure early recognition in such incidents the Center for Disease Control (CDC) actively encouraged the use of TCBS (thiosulfate-citrate-bile salt-sucrose) medium in searching for vibrios in diarrheal stools. At about the same time, published reports demonstrated the ubiquity of *V. parahae-*

molyticus in marine environments (5–10). Investigators at the Food and Drug Administration and in various state health and university laboratories were quick to recognize the public health potential of this disease in the United States. Within a few years, this organism became recognized as an important cause of foodborne disease in the United States and other countries.

ORGANISM

Vibrio parahaemolyticus is a halophilic, mesophilic, gram-negative, motile, facultative anaerobe found in marine fauna and marine water throughout the world. Its natural habitat is coastal and estuarian waters, where it lives in sediment during the cold winter months. As the temperature rises in late spring, the organism leaves the sediment and colonizes animal life. A direct relationship has been demonstrated between ambient temperature and abundance of the organism (9). *V. parahaemolyticus* has been recovered from a variety of seafoods, especially shellfish and crustaceans. Although in most instances a commensal relationship exists, the organism may cause disease in crabs and shrimp (11, 12).

V. parahaemolyticus is one of several marine vibrios whose taxonomic position is not yet entirely clear. Complete morphologic, physiologic, and biochemical characterization are necessary to confirm the diagnosis. Methods of isolating and identifying have been described (13). Seafood and stool specimens must be cultured on TCBS or on thymol-blue Teepol agar (BTP Teepol) or other media that support the growth of halophilic organisms; *V. parahaemolyticus* will not grow on most routine enteric media. For seafood specimens an enrichment procedure utilizing glucose-salt-Teepol broth or bismuth-sulfite-salt broth known as BS broth A (Eiken) is recommended, but fecal specimens can be inoculated directly on TCBS agar. The organism has characteristic morphology on TCBS and BTP Teepol, appearing as round smooth turbid colonies, 3 to 5 mm in diameter, with green or blue centers. Other features that help distinguish it from similar marine vibrios are its ability to grow at 43°C, failure to grow in 10% NaCl broth, and failure to ferment sucrose.

Although antigen typing is not considered a routine laboratory procedure, the organism can be serotyped by agglutination with type-specific somatic antisera. There are 11 recognized "O" groups and 52 "K" types, but many are untypable (13). H antigens of all types are serologically identical and are therefore not useful taxonomically. Other marine vibrios can agglutinate in *V. parahaemolyticus* O and K antisera; therefore serologic studies, while useful for taxonomic and epidemiologic purposes, are of limited diagnostic value.

V. parahaemolyticus isolates produce a number of distinct hemolysins. One of these substances produces the "Kanagawa" phenomenon (named for the prefecture in Japan where it was discovered), a specific in vitro hemolytic reaction that correlates with pathogenicity (3).

Cary-Blair and other general enteric transport media, except those containing glycerin, are suitable for field studies (14, 15). It is advisable to add 2–4% NaCl to transport media and other culture media used to transport, isolate, or identify this organism.

ENTEROPATHOGENESIS

The enteropathogenicity of *V. parahaemolyticus* in man has been established through extensive clinical and epidemiologic observations and a limited number of experimental studies. Pathogenic mechanisms have been studied in various animal models.

Acute gastroenteritis has been induced by feeding volunteer subjects broth cultures of isolates obtained from diarrheal stools (16, 17). Systematic study to define infective doses has not been reported, but an inverse relationship between size of inoculum and length of incubation period has been established. Two cases following accidental ingestion of organisms in the laboratory provide further direct evidence of pathogenicity in man (17, 18).

Extensive observations in Japan have demonstrated a lack of correlation between serotype and pathogenic potential of a given strain (19). This led to an initial impression that all strains might be pathogenic; however, in 1965 it was observed that isolates from diarrheal stools could be clearly differentiated from the majority of nonhuman isolates with respect to the Kanagawa hemolytic reaction (17). In a study in Japan, 96% of 2720 isolates from patients were Kanagawa-positive, while only 1% of 650 isolates from sea water and seafood were positive (20). Isolates from cases from other parts of the world, including the United States, have generally given a positive result when tested for the Kanagawa reaction, while almost all isolates obtained from marine environments and seafood, including a series of over 600 from many parts of the United States, have been Kanagawa-negative (21). The strains that produced disease in the volunteer studies and in the 2 reported laboratory-acquired infections were all Kanagawa-positive. None of the 15 volunteers who ingested 10^9 or more organisms from broth cultures of several different Kanagawa-negative strains developed gastroenteritis (20).

Attempts to induce transformation of Kanagawa reactivity by transfer of genetic material from Kanagawa-negative to Kanagawa-positive organisms and by passage of Kanagawa-negative organisms through the intestinal tracts of humans and animals, including germ-free mice, have all yielded negative results (17).

Broth cultures of isolates from patients' stools caused disease in dogs and cats (16). Intestinal disease was also induced in monkeys and kittens fed cell-free filtrates from broth cultures (22).

A series of studies using ligated rabbit ileal loop techniques for assessing pathogenicity have yielded mixed results. In several early studies with the classic ileal loop model of De & Chatterjee (23), suspensions of both Kanagawa-positive and -negative vibrios caused loop dilation (24). Subsequent experiments utilizing a modified ileal loop model have yielded high rates of loop positivity with suspensions of Kanagawa-positive cells but very low rates of positivity with Kanagawa-negative cells (25). In histologic studies of rabbit ileal loop tissue following exposure to broth cultures, there was evidence of bacterial invasion of the epithelium accompanied by an inflammatory reaction (26, 27).

Evidence of enterotoxin activity has been sought using culture supernates, cell wall preparations, and Kanagawa hemolysin. One investigator reported ileal loop fluid accumulation following inoculation of supernates from cultures of Kanagawa-positive and -negative cells (R. Sakazaki, personal communication, Dec. 1972), but several others reported a consistent lack of ileal loop reactivity to supernate preparations of all strains tested, regardless of Kanagawa reactivity (25). One study showed evidence of ileal loop reactivity to heated cell or cell wall preparations (25). Two groups have reported absence of ileal loop reactivity to inocula of purified Kanagawa hemolysin (17, 28).

EPIDEMIOLOGY

Knowledge of the magnitude and geographic distribution of disease caused by *V. parahaemolyticus* has been highly dependent on the utilization of halophilic culture media. In Japan, where these media have been widely used since the early 1960s, several thousand cases are reported annually. Since these techniques were widely introduced into the United States in the late 1960s, 13 common-source outbreaks and a number of sporadic cases have been reported (29). These outbreaks have been reported from Atlantic, Pacific, and Gulf Coast states and Hawaii and have involved a variety of seafoods including crab, shrimp, lobster, and oysters. A suspected outbreak occurred in Washington State in 1969, but the first full laboratory documentation of the disease in the United States was reported in Maryland in 1971.

Sporadic cases and outbreaks have been documented in Australia (30), the Philippines (31), South Vietnam (32), Thailand, Malaysia (18), India (33), Togo (34), Mexico, and Panama (personal communications). In England, disease caused by *V. parahaemolyticus* has been documented on two occasions in persons arriving by plane from Asian countries (35, 36).

Virtually all reported cases have occurred during warm months of the year. Epidemiologic evidence has almost always incriminated fish or seafood as the source of infection. Person-to-person transmission has not been observed (3, 29). In Japan, subclinical cases have been demonstrated in fewer than 1% of healthy individuals and approximately 7% of persons involved in preparing seafood in the summertime (37). Subclinical cases have not been detected during the winter months, suggesting that asymptomatic infection is a transient phenomenon.

CLINICAL FEATURES

V. parahaemolyticus gastroenteritis is usually a self-limited illness of moderate severity and short duration, but severe or protracted cases requiring hospitalization have occurred (33). Blood stream or visceral infections have not been noted. A few cases of wound infection acquired through direct contact with contaminated marine sources have been reported (R. Weaver, personal communication). Death has occurred in fewer than 1 per 1000 cases reported from Japan and in none of approximately 1000 cases reported in the United States.

The spectrum of signs and symptoms observed in a series of well-documented outbreaks in the United States is shown in Table 1 (29). Explosive watery diarrhea was the cardinal manifestation in over 90% of cases in most outbreaks. Nausea and cramping abdominal pain were the other most common manifestations, with vomiting in approximately 50% of cases. Fever, rarely exceeding 102°F, chills, and headache were reported less frequently. Duration of illness ranged from 2 hr to more than 10 days, with a median of 3 days. Median incubation periods for the series of outbreaks ranged from 13 to 23 hr, with incubation periods for individual cases ranging from 4 to 96 hr. No age or sex predilection has been noted.

Table 1 Clinical manifestations in eight laboratory-confirmed *Vibrio parahaemolyticus* outbreaks

Symptom	Incidence of Symptoms	
	Median	Range
Diarrhea	98%	80-100%
Abdominal cramps	82%	68-100%
Nausea	71%	40-100%
Vomiting	52%	17-79%
Headache	42%	13-56%
Fever	27%	21-33%
Chills	24%	4-56%

A dysenteric syndrome with mucoid or sanguinous stools has been described in a number of cases in Japan (3), India (33), Vietnam (32), and Australia (30), and in one isolated instance in the United States (38). Several cases with profuse rice-water stool have been reported from India (33) and Togo (34), but the profound fluid loss seen in cholera gravis has not occurred.

Diagnosis should be suspected on clinical and epidemiologic grounds when acute diarrhea develops following the ingestion of seafood. Confirmation depends upon recovery of the organism from stool and epidemiologically incriminated food; optimally, the culture should be obtained early in the course of illness. Commonly, multiple serotypes are isolated from a patient's stool, and frequently different strains are isolated from samples of incriminated food. Specific serotypes tend to predominate among clinical isolates in certain geographic areas and in certain years (39). Serotypically identical strains have been isolated from cases in different parts of the world (40). Methods of identifying circulating antibody in patient serum (16) and detecting organisms in stool or food specimens by fluorescent antibody (41) have been studied, but these are presently unreliable.

TREATMENT

The limited severity and duration of the illness militates against prescribing specific antimicrobial therapy or any other medication in most cases. However, in protracted or dysentery-like cases, antibiotic treatment should be prescribed. Anecdotal information suggests that tetracycline may be beneficial in such cases. The organism has been found sensitive in vitro to penicillin, tetracycline, chloramphenicol, streptomycin, kanamycin, polymyxin B, novobiocin, and oleandomycin (3, 42). In one study, patients treated with chloramphenicol, tetracycline, or kanamycin ceased to shed organisms within 5 days, while a number treated symptomatically continued to shed organisms for as long as 15 days. Information on clinical response to antibiotic treatment was not reported (26).

PREVENTION

Prevention of foodborne disease due to *V. parahaemolyticus* requires an awareness of two facts: (*a*) seafood acquired from coastal waters during warm months may be naturally contaminated with the organism and (*b*) a relatively small number of vibrios present in raw or undercooked seafood or in cooked seafood or other foods cross-contaminated from a raw source, may, under circumstances of inadequate refrigeration, proliferate to very large numbers in a relatively short time. Such potentially pathogenic levels of the bacterium generally do not make the seafood look or taste spoiled.

SUMMARY

As a well-established bacterial etiology of intestinal disease, *V. parahaemolyticus* possesses a number of unique attributes as well as several properties similar to those of other enteropathogens. It stands alone among foodborne pathogens in its exclusively marine ecology. Its potential presence either naturally in raw seafood or through cross-contamination of cooked food, with the capacity for proliferation to pathogenic levels in the absence of proper refrigeration, is analogous to nontyphoid salmonellae in poultry and meat products. Clinically, the intestinal infection caused by *V. parahaemolyticus* is usually very similar to that caused by salmonellae and some strains of enteropathogenic *Escherichia coli.* Occasionally, cases resemble bacillary dysentery or mild cholera. It is probable that serotypes differ in their pathogenic mechanisms; it appears that bacterial invasion of intestinal mucosa or elaboration of enterotoxin or both mechanisms may play a role under differing parasite and host circumstances. The relationship between the Kanagawa hemolytic reaction and enteropathogenicity appears to be a unique phenomenon. How Kanagawa positivity is conferred upon certain strains and what, if any, role it plays in pathogenicity remain unresolved.

Literature Cited

1. Fujino, T. et al 1953. *Med. J. Osaka Univ.* 4:299–304
2. Miyamoto, Y., Nakamura, K., Takizawa, K. 1961. *Jap. J. Microbiol.* 6:141–58
3. Sakazaki, R. 1969. *Foodborne Infections and Intoxications,* ed. H. Riemann, 115–29. New York: Academic
4. Gangarosa, E. J., Faich, G. A. 1971. *Ann. Intern. Med.* 74:412–15
5. Kampelmacher, E. H., Mossel, D. A. A., van Noorle Jansen, L. M., Vincentie, H. 1970. *J. Hyg.* 68:189–96
6. Thomson, W. K., Trenholm, D. A. 1971. *Can. J. Microbiol.* 17:545–49
7. Baross, J., Liston, J. 1970. *Appl. Microbiol.* 20:179–86
8. Fishbein, M., Mehlman, I. J., Pitcher, J. 1970. *Appl. Microbiol.* 20:176–78
9. Liston, J., Baross, J. 1973. *J. Milk Food Technol.* 36:113–17
10. Barrow, G. I., Miller, D. C. 1969. *Lancet* ii:421–23
11. Krantz, G. E., Colwell, R. R., Lovelace, E. 1969. *Science* 164:1286–87
12. Vanderzant, C., Nickelson, R., Parker, J. C. 1970. *J. Milk Food Technol.* 33: 161–62
13. Fishbein, M., Wentz, B. 1973. *J. Milk Food Technol.* 36:118–23
14. Neumann, D. A., Benenson, M. W., Hubster, E., Tuan, N. T. N. 1971. *Am. J. Clin. Pathol.* 57:33–34
15. Doki, C., Seol, S. Y., Ryunbin, T., Park, C. K. 1972. *Appl. Microbiol.* 24:675–78
16. Takikawa, I. 1958. *Yokohama Med. Bull.* 9:313–22
17. Sakazaki, R. 1972. *Proc. 8th Int. Symp. on Microbiol. Safety Food, 17–21 September, Reading, England*
18. Sanyal, S. C., Sil, J., Sakazaki, R. 1973. *J. Med. Microbiol.* 6:121–22
19. Sakazaki, R. 1965. *Proc. Cholera Res. Symp.* Public Health Service Pub. No. 1328, p. 30–34. Washington, D.C.: GPO
20. Sakazaki, R. et al 1968. *Jap. J. Med. Sci. Biol.* 21:325–31
21. Fishbein, M., Wentz, B., Landry, W. C., Maceachern, B. 1973. *Proc. Int. Symp. Vibrio Parahaemolyticus, 17–18 September, Tokyo, Japan*
22. Aiso, K., Matsuno, M. 1961. *Jap. J. Microbiol.* 5:337–46
23. De, S. N., Chatterjee, D. N. 1953. *J. Pathol.* LXVI:559–62
24. Sakazaki, R., Iwanami, S., Fukumi, H. 1963. *Jap. J. Med. Sci. Biol.* 16:161–83
25. Twedt, R. M., Brown, D. F. 1973. *J. Milk Food Technol.* 36:129–34
26. Kasai, G. J. 1971. *Southeast Asian J. Trop. Med. Public Health* 2:169–73
27. Yahagi, H., Ghoda, A., Sasaki, S. 1967. *Keio J. Med.* 16:119–32
28. Zen-Yoji, H., Hitokato, H., Morozumi, S., Le Clair, R. A. 1971. *J. Infec. Dis.* 123:665–67
29. Barker, W. H. 1973. *Proc. Int. Symp. Vibrio Parahaemolyticus, 17–18 September, Tokyo, Japan*
30. Battey, Y. M., Wallace, R. B., Allan, B. C., Keeffee, B. M. 1970. *Med. J. Aust.* 1:430–33
31. Sakazaki, R., Gomez, C. Z., Sebald, M. 1967. *Jap. J. Med. Sci. Biol.* 20:265–80
32. Neumann, D. A., Benenson, M. W., Hubster, E., Tuan, N. T. N., Van, L. T. 1972. *Am. J. Trop. Med. Hyg.* 21:464–66
33. Chatterjee, B. D., Gorbach, S. L., Neogy, K. N. 1970. *Bull. WHO* 42:460–63
34. Bockemühl, J., Amédomé, A., Triemer, A. 1972. *Z. Tropenmed. Parasitol.* 23: 308–15
35. Peffers, A. S. R., Bailey, J., Barrow, G. I., Hobbs, B. 1973. *Lancet* ii:143–45
36. Public Health Laboratory Service, United Kingdom. 1973. *Brit. Med. J.* 2: 562
37. Zen-Yoji, H. et al 1965. *J. Infec. Dis.* 115:436–44
38. Center for Disease Control. 1972. *Morbid. Mortal. Weekly Rep.* 21:245–46
39. Kudoh, Y., Ohta, K., Ikejima, N., Itoh, T., Zen-Yoji, H. 1970. *Ann. Rep. Tokyo Metrop. Res. Lab. Publ. Health* 22:15–20
40. Zen-Yoji, H., Le Clair, R. A., Ohta, K., Montague, T. S. 1973. *J. Infec. Dis.* 127: 237–41
41. Vanderzant, C., Nickelson, R. 1972. *Appl. Microbiol.* 23:26–33
42. Chatterjee, B. D., Neogy, K. N., Chowdhury, B. R. R. 1970. *Bull. WHO* 42: 640–41

Copyright 1974. All rights reserved

IMMUNOPATHOLOGY AND GLOMERULONEPHRITIS[1]

❖7097

Curtis B. Wilson, M.D. and Frank J. Dixon, M.D.
Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California

Immunopathologic studies begun in 1900 have resulted in the identification of a least two major mechanisms of immune glomerular injury, which appear to lead to over 85% of human glomerulonephritides (1). In both instances, the glomerular injury is induced by the interaction of antibodies with antigens, either in the glomerular basement membrane (GBM), so-called anti-GBM antibodies, or with non-glomerular antigens to form circulating nephritogenic immune complexes that deposit within the glomerular filter. Although cellular sensitivity may accompany the humoral immune response (2–4), there is little evidence that cellular immunity contributes significantly to the production of glomerulonephritis in contrast to transplant rejection where cellular immunity plays a prominent role.

Either type of antigen-antibody reaction (anti-GBM antibodies or immune complex deposition) within the glomerulus can set in motion a series of similar events that lead to tissue injury (5). The classical complement (C) sequence can be activated through $C1_q$ recognition of alterations in the immunoglobulin (Ig) molecule after its combination with antigen (6). During C activation, biologically active materials are released which include substances chemotactic (7–9) for polymorphonuclear leukocytes (PMNs). The PMNs thus drawn to the site of antigen-antibody reactions may be retained there by yet another property of C activation, namely immune adherence. PMNs that displace the endothelial cells and may even penetrate the GBM (10) have been shown to damage the surrounding glomerular substrate by release of lysozymal enzymes in experimental animals (11, 12) and presumably act similarly in man (13–15). Such enzymatic damage can be detected electron microscopically as lanthanum hydroxide (16) increasingly permeates the damaged GBM and fragments of GBM appear in the urine (17). Glomerular damage may also occur in experimental anti-GBM (18, 19) and immune complex (20, 21) glomerulonephritis in the absence of detectable C participation. In fact, roughly 25% of patients with anti-GBM antibody-induced glomerulonephritis do not have detectable glomerular-bound C (22) even though the IgG subclasses within the glomeruli have the intrinsic ability to activate C (23, 24). These observations suggest that pathogenic mechanisms independent of C may also produce glomerular damage under certain conditions. Studies are being directed to explore the pathogenic roles of vasoactive amines, kinins, and coagulation as well as their possible simultaneous involvement (25–28). Recent observations in patients with hypocomplementemic glomerulonephritis have suggested that nonimmune C activation by the recently described C3 proactivator system (29, 30) [probably identical with the properdin system (31)] may possibly induce or perpetuate glomerular damage as will be discussed in later sections.

---

[1] This is Publication No. 738 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California. This work was supported in part by United States Public Health Service Grant AI-07007 and United States Public Health Service Contract PH-43-68-621.

The utilization of similar mediators of immunologic injury by either antibody-induced form of glomerulonephritis probably explains the general lack of either histologic or clinical features that distinguish one from the other. Indeed, experimental rabbits with chronic serum sickness glomerulonephritis induced by daily injections of a single antigen developed virtually the entire spectrum of histologic forms of glomerulonephritis recognized in man (32). The histologic picture of chronic serum sickness with its attendant clinical course seems to be related to the tempo and magnitude of the immunologic insult. Similarly, in well-defined clinical entities such as systemic lupus erythematosus (SLE) or anti-GBM antibody-induced glomerulonephritis, there may be considerable histologic and clinical variations in spite of the fact that each entity has a characteristic immunologic basis.

The two antibody-induced mechanisms of glomerular injury can be differentiated readily by the immunofluorescent pattern of Ig deposition within the GBM. Anti-GBM antibodies react uniformly along the GBM to produce a smooth, linear deposition, whereas immune complexes deposit randomly along the GBM and in the mesangium to produce an interrupted granular pattern (33). Ultrastructural studies utilizing antibodies labeled with markers visible through the electron microscope have confirmed that anti-GBM antibodies combine with the GBM, presumably via endothelial fenestrations (34). Subendothelial, intramembranous, or most characteristically subepithelial electron-dense immune complex deposits can be shown by similar techniques to contain Ig, antigen, and C (35).

ANTI-GLOMERULAR BASEMENT MEMBRANE ANTIBODY-INDUCED GLOMERULONEPHRITIS

Experimental Anti-Glomerular Basement Membrane Nephrotoxicity

Historically, Lindeman demonstrated the nephrotoxicity of heterologous anti-kidney sera in 1900, and Masugi extended the studies in the 1930s. These and subsequent observations in experimental animals have been comprehensively reviewed (36). In brief, Kay recognized the role of the recipient's immune response to the heterologous nephrotoxic sera in the production of the autologous phase of nephrotoxic nephritis. In the 1950s, Krakower and Greenspon identified the GBM as a nephrotoxic antigen with cross-reactive antigens in other vascular tissues, particularly lung and placenta. More recently, quantitative studies have shown that 75 μg of heterologous kidney-fixing antibody per gram of kidney is required to produce immediate glomerular injury in a rat (1.2×10^{10} antibody molecules per glomerulus) (37), whereas only 5 μg per gram is effective in a sheep (38). Heterologous anti-GBM antibodies have been extremely important in studying the roles of C and PMNs in this form of glomerulonephritis; however, until recently such antibodies were not known to play a part in human disease. Heterologous anti-lymphocyte globulin (ALG) now used as an immunosuppressant in renal transplantation may contain antibodies cross-reactive with the GBM. Indeed, about 70% of ALG prepared by immunization with lymphocytes derived from solid lymphoid organs contains such antibodies (39) apparently induced by the inclusion of vascular and connective tissue fragments in the inoculum. The nephrotoxicity of these antibodies in human transplant recipients is difficult to assess due to concomitant immunosuppression and local absorption after intramuscular injection; however, some preparations contain sufficient antibodies to induce fatal glomerulonephritis in subhuman primates (40).

Experimental animals can also be induced to make antibodies cross-reactive with their own GBM by injecting GBM in adjuvant, thereby providing a convenient model in which to study anti-GBM glomerulonephritis in humans. In 1962, Steblay reported such an example in sheep given human GBM in adjuvant (41). The lesions were characterized by linear deposits of sheep Ig along their GBMs and circulating anti-GBM antibodies that became detectable after nephrectomy. These antibodies could be utilized to transfer glomerulonephritis to normal sheep, confirming their pathogenicity (38).

Autologous anti-GBM antibody production can also be initiated by injection of antigens that are isolated from normal urine and are cross-reactive with the GBM. About one half of rabbits so injected develop glomerular injury (42). Studies of the urinary GBM antigens in man have identified at least two acidic, carbohydrate-rich, high molecular weight antigens (43). Similar antigenic material is also present in the sera of rats and humans and may accumulate after nephrectomy, suggesting a urinary route of excretion for these materials which may be derived from basement membrane breakdown throughout the body (43,44). Variations in the tolerogenic and immunogenic potential of these materials may be important to the induction of anti-GBM antibody responses.

Anti-Glomerular Basement Membrane Antibody-Induced Glomerulonephritis in Man

In 1967, Lerner, Glassock & Dixon demonstrated the presence of anti-GBM antibodies in glomerulonephritic kidneys with linear Ig deposits. They confirmed the pathogenicity of these antibodies by transferring glomerulonephritis to subhuman primates with injections of anti-GBM antibodies isolated either from the serum or eluted from the kidneys of such patients. The final and most convincing evidence for the nephritogenic potential of anti-GBM antibodies came when glomerulonephritis was accidentally transferred to a renal allograft placed in a patient with circulating anti-GBM antibodies (45).

Anti-GBM antibodies appear to be responsible for about 5% of human glomerulonephritides, although the exact incidence is difficult to estimate. We have recently identified 63 such patients in a series of about 900 patients which included several patients sent specifically for identification of anti-GBM antibodies, probably artificially increasing the incidence (22).

Anti-GBM antibodies most often induce a rapidly progressive glomerulonephritis, although occasionally a more chronic histologic and clinical picture may ensue. In about half of the patients, pulmonary hemorrhage is associated with the renal disease to produce a clinical picture termed Goodpasture's syndrome (46, 47). Since pulmonary hemorrhage occasionally accompanies uremia or other systemic disease (48, 49), the term Goodpasture's syndrome should be reserved for those patients in whom anti-GBM antibodies can be demonstrated. For reasons that are poorly understood, young males are most commonly affected.

The pulmonary involvement in patients with anti-GBM antibody-associated Goodpasture's syndrome suggests a common immunopathogenesis. Linear deposits of Ig can be identified along the alveolar septum (50, 51), and GBM-reactive antibodies can be eluted from the lungs of these patients (52, 53). In experimental animals, passive transfer of anti-lung antibodies produces glomerulonephritis (44, 54), and lung tissue contains antigens capable of inducing autologous formation of antibodies reactive with GBM (55). Studies of the basement membrane reactivity of antibodies eluted from the kidneys of patients with Goodpasture's syndrome demonstrate a broader specificity than those from patients with renal disease only (53).

Little is known about the events that lead to nduction of spontaneous anti-GBM antibody production, partly because the only species other than man where spontaneous anti-GBM antibody-induced nephritis has been found is the horse (56). The presence of material in the serum and urine that is antigenically similar to the GBM suggests one antigenic source. Extravasation or enhanced immunogenicity of this antigen secondary to adjuvant effects of a concurrent infection potentially could lead to the production of anti-GBM antibodies. Alternately, cross-reactive material from the environment or exposure and/or alteration of self antigens by environmental factors might be involved. For example, several patients with Goodpasture's syndrome have had a history of hydrocarbon solvent inhalation (57), and at least two patients have had influenza A2 infections coincident with the onset of Goodpasture's syndrome (22, 58). In addition to causing possible immunologic alterations in the basement membrane structure, presumably in the lung, these pulmonary insults might expose alveolar antigens that can then react with anti-GBM antibodies induced by some other mechanism.

The GBM is composed of collagenous and noncollagenous proteins assembled in an as yet unidentified structure. It has been possible to remove the collagen portion of the GBM almost completely through collagenase digestion and dialysis, leaving noncollagenous proteins that contain nephritogenic antigen(s) capable of reacting with eluted anti-GBM antibodies (59). Nonenzymatic solubilization of the GBM with chaotropes (NaTCA, KCL, or KBr) releases varying portions of collagenous and noncollagenous proteins, and these contain differing amounts of nephritogenic antigen(s) capable of inhibiting the reaction of GBM with anti-GBM antibodies eluted from kidneys of patients with anti-GBM antibody-induced glomerulonephritis (60). Further separation of these fractions of GBM and inhibition of GBM reactivity of human anti-GBM antibodies in radioimmunoassay studies suggest that nephritogenic antigen(s) resides in the glycoprotein portion of the noncollagenous proteins (H. Marquardt, C. B. Wilson, and F. J. Dixon, unpublished observations).

Anti-GBM antibody-induced glomerulonephritis can be diagnosed tentatively by the immunofluorescent detection of linear deposits of Ig along the GBM. The specificity of the linear Ig deposit should be confirmed by elution of glomerular Ig (2–34 μg IgG/g of kidney) (22) and by subsequent in vitro or in vivo testing of the eluted Ig, since occasionally nonspecific linear deposits of Ig can be identified in kidneys obtained at autopsy, after perfusion prior to transplantation, and from patients with diabetes mellitus (22, 61–63). These latter deposits apparently reflect a nonimmunologic increase in the 1–2% of Ig normally present in the GBM (59). Alternately, circulating anti-GBM antibodies can be sought by indirect immunofluorescence (64), hemagglutination (3), or radioimmunoassay (59, 65). By indirect immunofluorescence, 87% of patients with anti-GBM antibody-associated Goodpasture's syndrome and 62% of patients with anti-GBM antibody nephritis can be shown to have circulating anti-GBM antibodies (22); the sensitivity of the radioimmunoassay in detecting circulating anti-GBM antibodies appears to be even greater (C. B. Wilson, H. Marquardt, and F. J. Dixon, unpublished observations). The duration of the anti-GBM antibody response varies from 3 to 25 months (mean 7 months) after nephrectomy preparatory to renal transplantation (22). It is currently unknown if nephrectomy is required for the disappearance of the anti-GBM antibody response.

Therapeutic considerations are beyond the scope of this review; however, nephrectomy and transplantation deserve mention. Bilateral nephrectomy has been proposed as beneficial if not life-saving for patients with Goodpasture's syndrome and severe pulmonary hemorrhage (66–68). A uniformly favorable response, however, has not been found since 6 of 19 Goodpasture's patients continued to have pulmonary hemorrhages after nephrectomy, contributing to death in 3 (22). Any beneficial effect of nephrectomy must reflect a balance between increased circulating anti-GBM antibodies as their target tissue is removed and a corresponding increase in circulating immunoabsorbents as circulating cross-reactive basement membrane antigens can no longer be excreted. As the radioimmunoassay methods are applied to quantitate both circulating basement membrane antigens and antibodies, the effects of nephrectomy will be better understood.

Severe anti-GBM antibody-induced nephritis usually results in renal failure (often rapidly) necessitating dialysis and/or transplantation. When transplantation has been postponed until circulating anti-GBM antibodies have disappeared, the response has been favorable (22, 67). Anti-GBM antibody glomerulonephritis, however, developed in 19, possibly 20, of 34 patients after transplantation. Twenty four of these patients had been known to have anti-GBM antibody-induced glomerulonephritis as their original disease, while the other 10 had no pretransplant immunopathologic characterizations. Marked glomerular damage resulted in graft failure in 7, possibly 8 instances (22). Severe recurrences seem to relate to the presence of circulating anti-GBM antibodies and short or no anephric periods prior to transplantation. Only 1 of 11 patients with undetectable circulating anti-GBM antibodies had a severe recurrence, suggesting that reactivation of the anti-GBM response after transplantation may occasionally occur.

IMMUNE COMPLEX-INDUCED GLOMERULONEPHRITIS

Experimental Immune Complex Glomerulonephritis

The glomerular injury that accompanies serum sickness is the classic example of immune complex glomerulonephritis. VonPirquet in 1911 recognized the relationship between the host's immune response to foreign serum protein and the development of serum sickness. This observation and the extensions by Longcope, Rich & Gregory, and Hawn & Janeway have been reviewed recently (69). During the 1950s, the work of Dixon (32, 70, 71) and others (72–74) established the immune complex as a toxic product of the immune reaction as envisioned by VonPirquet years before. The concept is best understood by examining the features attendant to the development of acute serum sickness in rabbits induced by a single intravenous injection of 750 mg of radiolabeled bovine serum albumin (BSA). The BSA rapidly equilibrates with the intra- and extravascular fluids and then disappears at a rate governed by its catabolic half-life (T-½) of about four days. Antibody production begins, and since antigen remains in the circulation, immune complexes form. Initially antigen is present in great excess so that the complexes are small (Ag_2Ab), fix C poorly, and continue to circulate. As the amount of antibody produced increases, the composition of the immune complexes shifts away from antigen excess toward equivalence. They can fix C and are eliminated from the circulation largely via phagocytosis by the reticuloendothelial system. During this period of immune elimination a small fraction of the immune complexed antigen deposits in vessels, including glomeruli, thereby inciting a phlogogenic response. During the several days of acute serum sickness only about 20 μg BSA in immune complex form deposits within the total glomerular mass (4.4×10^8 molecules/glomerulus) as measured by tracing radioiodinated BSA (75). The sensitivity of the immunofluorescent technique in detecting the BSA in glomeruli can be quantitated to be 0.25 μg BSA/g of kidney. The free antibody which appears in the circulation after immune elimination continues to deposit on the glomerular-bound BSA (76), soon rendering it undetectable by the immunofluorescent technique while deposits of Ig and C actually increase (75, 77). This covering of the free antigenic determinants of the glomerular-bound immune complex by circulating antibody may explain in part the difficulty encountered in identifying antigens in human immune complex glomerulonephritis. The glomerular lesion of acute serum sickness resolves rapidly, usually without permanent structural damage, consistent with the observation that neither C nor PMNs are required for its production (20, 21).

By giving daily intravenous injections of BSA to rabbits in amounts to balance antibody production, a chronic serum sickness glomerulonephritis can be induced that greatly resembles human chronic glomerulonephritis (32). When rabbits with brisk immune responses are given large amounts of antigen, an active, proliferative, necrotizing, crescent-forming glomerulonephritis develops in contrast to a membranous histologic picture in rabbits that have poor immune responses and require only small amounts of antigen to balance their antibody production. If doses of antigen are purposely given to favor either antibody excess or extreme antigen excess, nephritogenic immune complexes do not form. Immune complexes formed in moderate antigen excess are the most nephritogenic. The avidity of the antibody may influence the nephritogenic potential of immune complexes in certain instances when rabbits are given fixed small daily doses of antigen which favor formation of deleterious immune complexes only in poor antibody producers (78, 79); however, when appropriate doses are selected, glomerulonephritis can be induced in any rabbit that forms antibody (80). In these instances, antibody avidity increases concomitantly with the onset of proteinuria (C. B. Wilson and F. J. Dixon, unpublished observations).

The use of radiolabeled BSA has allowed the quantitation of immune complexed antigen in kidneys from rabbits with chronic BSA-induced serum sickness. During the two-month developmental phase of chronic serum sickness glomerulonephritis, only about 0.04% of the injected dose of 10–200 mg deposits daily. Somewhat before or coincident with the onset of proteinuria, the daily deposition increases to about 0.5% of the daily dose (80). A shifting of BSA deposits from the mesangium to the GBM coincident with the onset of proteinuria suggests that the phagocytic role of the mesangial cell may be protective but is exhaustible. No quantitative studies of mesangial function are available in this model; however, functions of the total reticuloendothelial system appear to be low at the onset of proteinuria (80). Structural studies also suggest that mesangial hypertrophy may be found early in immune complex glomerulonephritis, and it is related by some to the presence of large immune complexes (81).

Factors responsible for immune complex deposition in glomeruli have been the subject of study. The role of vasoactive substances in the vascular deposition of immune complexes (82, 83) and of other inert substances such as colloidal carbon has been recognized (84). The mechanisms of vasoactive amine release including the platelet-activating factor derived from basophils after the reaction of BSA with IgE antibody adhering to the basophil surface have been recently reviewed (85). Administration of vasoactive amine antagonists has lessened the severity of acute (86) and possibly chronic (87, 88) serum sickness glomerulonephritis in rabbits. In addition, the size of the circulating immune complex appears to be critical with complexes greater than 19S localizing in both acute (89) and chronic (80) serum sickness. Hydrodynamic forces are also involved as shown by increased deposition of complexes in vascular bifurcations (72, 86) and during experimental hypertension (90).

The T-½ of glomerular-bound immune complexed BSA is about ten days (75) in rabbits with acute serum sickness and about five days (80) in rabbits injected daily with BSA in chronic serum sickness. A beneficial effect of administration of huge excesses of antigen has been noted (69, 91) in chronic serum sickness; in fact, the

T-½ of glomerular-bound immune complexed antigen can be shortened to one day (80). In addition, huge excesses of antigen appear to terminate antibody production (C. B. Wilson and F. J. Dixon, unpublished observations), thereby providing an additional therapeutic benefit. Indeed, if the antigens of human immune complex glomerulonephritis were known and available in a pure nontoxic form, their administration could provide an immunologically specific therapy.

Experimental and spontaneous immune complex glomerulonephritis in animals may be caused by exogenous antigens other than foreign proteins as well as by endogenous antigens. Chronic persistent viral infections such as lymphocytic choriomeningitis, lactic dehydrogenase virus, equine infectious anemia, Aleutian disease of mink, and spontaneous murine leukemias are frequently associated with immune complex glomerulonephritis (92–104) and are typified by circulating viral-antigen-antibody complexes and granular deposits of Ig (anti-viral antibody), C3, and viral antigen in glomeruli. The large number of virally induced immune complex glomerulonephritides in animals suggests that viral antigens are likely candidates for agents that produce immune complex glomerulonephritis in humans. Both antiviral antibodies (105, 106) and antinuclear "auto" antibodies (107) can be found in the glomerulonephritis that occurs spontaneously in New Zealand mice, a disease similar in many aspects to systemic lupus erythematosus in humans. Acceleration (108, 109) or retardation (110) of the course of glomerulonephritis in these mice by purposefully infecting them with viral agents also suggests the potential association of viral infections with the course of "autoimmune" immune complex diseases. Immune complex glomerulonephritis can also be induced with homologous renal tubular antigens and adjuvant in rats (111, 112), with thyroglobulin in rabbits (113, 114), with histocompatibility antigens in graft-versus-host reactions in mice (115), and occurs spontaneously with erythrocyte antigens in mice (116, 117).

Immune Complex-Induced Glomerulonephritis in Man

By immunofluorescence, up to 80% of human glomerulonephritic kidneys have granular glomerular deposits of Ig and C3, suggesting immune complex deposition (1). Distribution of the glomerular deposits may be either coarse or finely granular and present diffusely along the GBM or in a focal segmental arrangement. In some instances the deposition is limited to the mesangium. These patterns of deposition appear to correspond to diffuse, focal, and mesangial histologic alterations (33, 81, 118). Renal biopsies from patients with proteinuria and nephrotic syndrome and the minimal change lesion of lipoid nephrosis as a rule do not contain IgG, IgA, IgM, or C3; a recent demonstration of IgE in some of these patients (119) has not been confirmed in other studies (120). Attempts are being made to divide presumed immune complex glomerulonephritis into subclassifications according to the different Ig classes deposited within glomeruli. For example, some nephrologists have suggested that a focal glomerulonephritis characterized by largely mesangial IgA deposits, usually along with IgG and C3, represents a distinct glomerulopathy (121, 122). Nevertheless, in our experience and that of others (123, 124), IgA deposits are frequently found in many forms of glomerulonephritis, including those with Schönlein-Henoch purpura and lupus erythematosus.

Unfortunately, no simple and sensitive method is available to detect circulating immune complexes in contrast to anti-GBM antibodies. Analytical and sucrose density gradient ultracentrifugation and gel filtration are insensitive methods and not clinically useful. Interactions of immune complexes with $C1_q$ (125) and with monoclonal rheumatoid factors (126, 127) offer promise as screening tests. Detection of immune complexes bound to leukocytes (128–131) and studies of cryoprecipitable proteins (Ig-C complexes) sometimes present in patients with glomerulonephritis (132–135) may also be helpful.

Although a number of antigen-antibody systems have been identified in human immune complex glomerulonephritis, the antigens involved in the majority of cases remain unknown. As in experimental animals both exogenous and endogenous antigens have been implicated. The administration of foreign (exogenous) antisera, drugs, or immunization regimens may potentially induce nephritogenic immune complexes (136–139). More often, antigens derived from bacterial, protozoan, and viral agents have been implicated. The immunofluorescent pattern of granular Ig and C3 deposits (140–142) and subepithelial electron-dense deposits seen by electron microscopy (143–145) strongly implicate an immune complex etiology for the acute proliferative glomerulonephritic lesion (146) that follows nephritogenic streptococcal infections. While glomerular-bound streptococcal cell surface products (147) have been identified inconsistently in the past (148), they have not conformed to the extent or precise localizations of the accompanying Ig deposits. More recently, Treser et al have shown that, as in acute serum sickness in rabbits (149), the streptococcal antigen can be identified only early in the course of disease and is apparently rendered undetectable later by deposits of circulating antibody that persist in the glomeruli for some time. An alternate immunopathogenic mechanism of streptococcal glomerulonephritis has been favored by some investigators who propose that antibodies cross-reactive between streptococcal and GBM antigens could be involved (150, 151). Such a mechanism, however, would not be consistent with the immunofluorescent and electron microscopic observations that suggest an immune complex etiology.

Another presumed bacterial antigen-associated immune complex-induced glomerulonephritis is that which accompanies infection of ventriculo-atrial shunts used in the management of hydrocephalic children (152, 153). In one such case, staphylococcal antigens identical with those from the bacteria isolated from the infected shunt were identified in granular immune complex deposits (154). Other probable bacterial immune complex-induced glomerular lesions are those typified by granular Ig, C3, and/or electron-dense deposits in patients with bacterial endocarditis (155, 156) and syphilis (157–159). The nephrosis of African children suffering from quartan malaria is characterized by glomerular immune complex deposits containing plasmodial antigens and antibodies detectable by immunofluorescence and elution (160–162). The potential usefulness of identifying causative agents in presumed glomerular immune complex deposits in diseases of obscure etiology such as sarcoidosis should be considered when granular deposits of Ig and C3 can be identified within glomeruli of such patients (163).

Viral infections of animals are associated commonly with immune complex glomerulonephritis, and although in man viral agents can cause glomerulonephritis, none has been identified as a common cause either by serum antibody screening (164) or direct isolation from renal biopsies (165). Australian antigen has been found in the glomerulonephritic lesion (166) infrequently associated with hepatitis (167). Measles antigens have been detected by immunofluorescence in the glomeruli of a patient with subacute sclerosing panencephalitis (168). Oncornaviral-related antigens have been tentatively localized in glomerular immune complex deposits in patients with lymphoma and leukemia (169), and in kidneys immune complex deposits are being used in attempts to identify viral agents in patients with lung and other solid organ neoplasms. Other viral antigen-antibody systems will surely be found when glomerular lesions accompanying viral infections such as infectious mononucleosis are studied in detail (170).

The best example of apparently endogenous antigens identified in human immune complex glomerulonephritis is in SLE, where anti-nuclear antibodies including anti-DNA antibodies in immune complex form were correlated with glomerular deposits of Ig, C3, and DNA (85). Electron-dense deposits can be identified in subepithelial, intramembranous, and commonly subendothelial positions by electron microscopy. Anti-nuclear antibodies including those reactive with both native and single-stranded DNA, nucleoprotein, and phosphate buffer-extractable nuclear antigens (171) can be identified in eluates from glomerulonephritic lupus kidneys. Additional antigen-antibody systems such as those of rheumatoid factors (172) and mixed 19S-7S cryoglobulins (171) may also play a role in the glomerular immune complex deposition. The many recent reports of virus-like structures in vascular endothelial cells of the kidney and skin as well as in peripheral lymphocytes (173, 174) found in lupus and other patients coupled with the reported elevations of anti-viral antibody titers of as yet undetermined significance (164) suggest that, as in NZB mice, viral infections may have a poorly understood etiologic role in patients presumably genetically predisposed to autoantibody formation.

Other endogenous antigen-antibody systems can be involved in immune complex glomerulonephritis in humans. Thyroglobulin-antithyroglobulin complexes have been found in glomerular lesions of patients with thyroiditis (175) (R. Schwartz, personal communication). Nephritogenic immune complexes apparently form when antibody levels are reduced by immunosuppression, or circulating antigen is increased during therapeutic thyroid injury. Experimentally, thyroiditis and production of antithyroglobulin antibodies can be induced in rabbits by immunization with heterologous or chemically altered homologous thyroglobulin (113). These antibodies can interact with circulating thyroglobulin, especially when the latter is released secondary to thyroid injury, to cause glomerulonephritis with glomerular deposits of immunoglobulin, C3, and thyroglobulin (114). Recently three Japanese patients with membranous glomerulonephritis were reported to have glomerular immune complex deposits of Ig and renal tubular antigens (176) similar to those identified in the autologous immune complex nephritis induced in rats by injection of brush border antigen in adjuvant (112). Mercuric chloride-induced tubular injury can also be associated with glomerulonephritis suggesting an autoimmune response to tubular antigens (177), possibly bearing a relationship to the disease of the Japanese patients. A similar circulating immune response to renal tubular antigens has been observed in a patient after transplantation (C. B. Wilson, unpublished observations), and such a response can also be induced by successive implantations of unvascularized Lewis rat kidneys placed in peritoneal cavities of brown Norway rats (178). Other possible human immune complex glomerulonephritides have been identified, for example, in patients, usually children, with anaphylactoid purpura (Schönlein-Henoch syndrome). Granular deposits of Ig, C3, and fibrin are present in the mesangium and GBM (179–181). The antigenic content of the presumed immune complexes has not been identified; however, when the condition begins after an infection, microbial agents would be suspected.

Immune complex glomerulonephritis often results in renal failure where transplantation may be desirable. As with anti-GBM antibody-induced glomerulonephritis, the transplant in a patient with ongoing immune complex glomerulonephritis often is subject to immune glomerular injury by the original nephritogenic mechanism. Indeed, the development of glomerular injury may limit the usefulness of transplantation even after the problem of rejection has been circumvented. Recurrence of immune complex glomerulonephritis cannot be documented with certainty until identical antigen-antibody systems are identified in both native and transplanted kidneys as was done with anti-GBM antibodies. The recurrence of presumed immune complex glomerulonephritis in a transplant from an identical twin, where rejection would not be expected, certainly suggests recurrence, however (182). Recurrence of autologous immune complex nephritis in syngenic rat transplants has been documented (183). Alternately, immune responses to the allograft (184, 185) could produce immune complex injury as suggested by the glomerulonephritis accompanying graft-versus-host reactions in mice (115), and immune responses to renal tubular antigens have been induced by transplantation in both rats and rabbits (178). We have also observed the apparent de novo formation of anti-renal tubular basement membrane antibodies in two patients after transplantation (C. B. Wilson, unpublished observations). In any case, it would seem unwise to expose the renal homograft to an environment of potential nephritogenic immune complexes, particularly when the initial clinical course has been explosive. Until better methods for detecting circulating immune complexes are available, however, no scientific guidelines can be laid down for optimum timing of renal transplantation in accord with the nephritogenic potential of the patient.

Hypocomplementemic Glomerulonephritis

One form of glomerulonephritis most frequently found in children is associated with persistent hypocomplementemia and granular deposits of C3 often accompanied by Ig in the glomeruli (186–188). Selective depression in serum C3 and terminal C component levels (189–191) as well as depressed serum properdin levels (192) and glomerular properdin deposits (193) suggests activation of the C sequence at the C3 level, presumably utilizing the properdin and probably identical C3 proactivator systems (29–31). The concept of possible nonimmune activation of C by this system is supported by the finding that some of the patients have circulating factors (194) that persist after nephrectomy (195) and are capable of activating C3 in vitro. There is, however, no current evidence to relate the C activation with the pathogenesis of the glomerular injury. Indeed, prolonged daily activation of the alternate C pathway in rabbits with inulin or zymosan has failed to induce selective C deposition within glomeruli (196).

Roughly 10% of patients, usually those with far advanced glomerulonephritis, have glomerular C3 and terminal C component deposits without detectable Ig, $C1_q$, or C4 (197). In some patients a transition can be identified in adjacent glomeruli in which small amounts of Ig, $C1_q$, and C4 are associated with larger amounts of C3, C5, C6, and C8, while the majority of glomeruli contain only C3 and later C components (197). This observation suggests a change in the pattern of C activation within the glomerulus presumably initiated by immune complex deposition and perpetuated by factors active through the alternate C pathway. For example, bound $C3_b$ may activate the alternate pathway by a feedback mechanism (30). Investigations into these mechanisms and into the antigenic content of glomerular-bound immune complexes are among the most promising in immunopathologic nephrology.

Literature Cited

1. Wilson, C. B., Dixon, F. J. 1974. *Controversy in Internal Medicine II.* Philadelphia: Saunders
2. Rocklin, R. E., Lewis, E. J., David, J. R. 1970. *N. Engl. J. Med.* 283:497–501
3. Mahieu, P., Dardeene, M., Bach, J. F. 1972. *Am. J. Med.* 53:185–92
4. Dardeene, M., Zabriskie, J., Bach, J. F. 1972. *Lancet* 1:126–28
5. Cochrane, C. G. 1968. *Advan. Immunol.* 9:97–162
6. Müller-Eberhard, H. J. 1971. *Progress In Immunology,* ed. B. Amos, 553–65. New York: Academic
7. Ward, P. A., Cochrane, C. G., Müller-Eberhard, H. J. 1966. *Immunology* 11: 141–53
8. Ward, P. A. 1967. *J. Exp. Med.* 126: 189–206
9. Ward, P. A., Newman, L. J. 1969. *J. Immunol.* 102:93–99
10. Burkholder, P. M. 1969. *Am. J. Pathol.* 56:251–65
11. Cochrane, C. G., Aikin, B. S. 1966. *J. Exp. Med.* 124:733–52
12. Weissmann, G. 1967. *Ann. Rev. Med.* 18:97–112
13. Janoff, A., Scherer, J. 1968. *J. Exp. Med.* 128:1137–55
14. Janoff, A., Zeligs, J. D. 1968. *Science* 161:702–4
15. Lazarus, G. S., Brown, R. S., Daniels, J. R., Fullmer, H. M. 1968. *Science* 159: 1483–85
16. Gang, N. F., Trachtenberg, E., Allerhand, J., Kalant, N., Mautner, W. 1970. *Lab. Invest.* 23:436–41
17. Hawkins, D., Cochrane, C. G. 1968. *Immunology* 14:665–81
18. Unanue, E. R., Dixon, F. J. 1964. *J. Exp. Med.* 119:965–82
19. Henson, P. M. 1971. *Progress In Immunology,* ed. D. B. Amos, 155–71. New York: Academic
20. Kniker, W. T., Cochrane, C. G. 1965. *J. Exp. Med.* 122:83–97
21. Henson, P. M., Cochrane, C. G. 1969. *Cellular and Humoral Mechanisms in Anaphylaxis and Allergy,* ed. H. Z. Movat, 129–43. Basel: Karger
22. Wilson, C. B., Dixon, F. J. 1973. *Kidney Int.* 3:74–89
23. Lewis, E. J., Busch, G. J., Schur, P. H. 1970. *J. Clin. Invest.* 49:1103–13
24. McPhaul, J. J., Jr., Dixon, F. J. 1971. *J. Immunol.* 107:678–84
25. McCluskey, R. T., Vassalli, P., Gallo, G., Baldwin, D. S. 1966. *N. Engl. J. Med.* 274:695–701
26. Cochrane, C. G., Wuepper, K. D. 1971. *J. Exp. Med.* 134:986–1004
27. Henson, P. M., Cochrane, C. G. 1971. *J. Exp. Med.* 133:554–71
28. Zimmerman, T. S., Müller-Eberhard, H. J. 1971. *J. Exp. Med.* 134:1601–7
29. Götze, O., Müller-Eberhard, H. J. 1971. *J. Exp. Med.* 134:90s–108s
30. Müller-Eberhard, H. J., Götze, O. 1972. *J. Exp. Med.* 135:1003–8

31. Götze, O., Müller-Eberhard, H. J. 1973. *J. Immunol.* 111:288
32. Dixon, F. J., Feldman, J. D., Vazquez, J. 1961. *J. Exp. Med.* 113:899–920
33. Wilson, C. B. *Textbook of Immunopathology,* ed. P. A. Miescher, H. J. Müller-Eberhard. New York: Grune & Stratton. In press
34. Druet, P., Bariety, J., Bellon, B., Laliberte, F. 1972. *Lab. Invest.* 27: 157–64
35. Andres, G. A., Seegal, B. C., Hsu, K. C., Rothenberg, M. S., Chapeau, M. L. 1963. *J. Exp. Med.* 117:691–704
36. Unanue, E. R., Dixon, F. J. 1967. *Advan. Immunol.* 6:1–90
37. Unanue, E. R., Dixon, F. J. 1965. *J. Exp. Med.* 121:697–714
38. Lerner, R., Dixon, F. J. 1966. *J. Exp. Med.* 124:431–42
39. Wilson, C. B., Dixon, F. J., Fortner, J. G., Cerilli, J. 1971. *J. Clin. Invest.* 50: 1525–35
40. Taylor, H. E. 1970. *Transplant. Proc.* 2:413–16
41. Steblay, R. W. 1962. *J. Exp. Med.* 116: 253–71
42. Lerner, R., Dixon, F. J. 1968. *J. Immunol.* 100:1277–87
43. McPhaul, J. J., Jr., Dixon, F. J. 1969. *J. Exp. Med.* 130:1395–1409
44. Willoughby, W. F., Dixon, F. J. 1970. *J. Immunol.* 104:28–37
45. Lerner, R., Glassock, R. J., Dixon, F. J. 1967. *J. Exp. Med.* 126:989–1004
46. Benoit, F. L., Rulon, D. B., Theil, G. B., Doolan, P. D., Watten, R. H. 1964. *Am. J. Med.* 37:424–44
47. Proskey, A. J., Weatherbee, L., Easterling, R. E., Greene, J. A., Weller, J. M. 1970. *Am. J. Med.* 48:162–73
48. Martinez, J. S., Kohler, P. F. 1971. *Ann. Intern. Med.* 75:67–76
49. Lewis, E. J., Schur, P. H., Busch, G. J., Galvanek, E., Merrill, J. P. 1973. *Am. J. Med.* 54:507–13
50. Beirne, G. J., Octoviano, G. N., Kopp, W. L., Burns, R. O. 1968. *Ann. Intern. Med.* 69:1207–12
51. Markowitz, A. S., Battifora, H. A., Schwartz, F., Aseron, C. 1968. *Clin. Exp. Immunol.* 3:585–92
52. Koffler, D., Sandson, J., Carr, R., Kunkel, H. G. 1969. *Am. J. Pathol.* 54: 293–305
53. McPhaul, J. J. Jr., Dixon, F. J. 1970. *J. Clin. Invest.* 49:308–17
54. Hagadorn, J. E., Vazquez, J. J., Kinney, T. R. 1969. *Am. J. Pathol.* 57:17–30
55. Steblay, R. W., Rudofsky, U. 1968. *Science* 160:204–6
56. Banks, K. L., Henson, J. B. 1972. *Lab. Invest.* 26:708–15
57. Beirne, G. J., Brennan, J. T. 1972. *Arch. Environ. Health* 5:365–69
58. Wilson, C. B., Smith, R. C. 1972. *Ann. Intern. Med.* 76:91–94
59. Marquardt, H., Wilson, C. B., Dixon, F. J. 1973. *Kidney Int.* 3:57–65
60. Marquardt, H., Wilson, C. B., Dixon, F. J. 1973. *Biochemistry* 12:3260–65
61. Koffler, D., Agnello, V., Carr, R. I., Kunkel, H. G. 1969. *Am. J. Pathol.* 56:305–16
62. Gallo, G. R. 1970. *Am. J. Pathol.* 61: 377–94
63. Westberg, N. G., Michael, A. F. 1972. *Diabetes* 21:163–74
64. McPhaul, J. J. Jr., Dixon, F. J. 1969. *J. Immunol.* 103:1168–75
65. Mahieu, P., Lambert, P. H. 1973. *Fed. Proc.* 32:827
66. Maddock, R. K., Stevens, L. E., Reemtsma, K., Bloomer, H. A. 1967. *Ann. Intern. Med.* 67:1258–64
67. Halgrimson, C. G. et al 1970. *Arch. Surg.* 103:283–89
68. Nowakowski, A. et al 1971. *Ann. Intern. Med.* 75:243–50
69. Dixon, F. J. 1963. *Harvey Lect.* 58: 21–52
70. Dixon, F. J. 1962. *Second International Symposium on Immunopathology,* 71–87. Basel: Benno Schwabe
71. Dixon, F. J., Vazquez, J. J., Weigle, W. O., Cochrane, C. G. 1958. *Arch. Pathol.* 65:18–28
72. Germuth, F. G. 1953. *J. Exp. Med.* 97: 257–82
73. Germuth, F. G., Flanagan, C., Montenegro, M. R. 1957. *John Hopkins Med. J.* 101:149–69
74. Germuth, F. G., McKinnon, G. E. 1957. *Johns Hopkins Med. J.* 101: 13–42
75. Wilson, C. B., Dixon, F. J. 1970. *J. Immunol.* 105:279–90
76. Lambert, P. H., Miescher, P. A. 1972. *Fed. Proc.* 31:79
77. Fish, A. J., Michael, A. F., Vernier, R. L., Good, R. A. 1966. *Am. J. Pathol.* 49:997–1022
78. Pincus, T., Haberkern, R., Christian, C. L. 1968. *J. Exp. Med.* 127:819–32
79. Christian, C. L. 1969. *N. Engl. J. Med.* 280:878–84
80. Wilson, C. B., Dixon, F. J. 1971. *J. Exp. Med.* 134:7s–18s
81. Germuth, F. J. Jr., Rodriguez, E. 1973.

Immunopathology of the Renal Glomerulus. Boston: Little Brown
82. Cochrane, C. G. 1963. *J. Exp. Med.* 118:489–502
83. Cochrane, C. G. 1963. *J. Exp. Med.* 118:503–13
84. Benacerraf, B., McCluskey, R. T., Patras, D. 1959. *Am. J. Pathol.* 35:75–91
85. Cochrane, C. G., Koffler, D. 1973. *Advan. Immunol.* 16:185–264
86. Kniker, W. T., Cochrane, C. G. 1968. *J. Exp. Med.* 127:119–36
87. Kniker, W. T. 1968. *Fed. Proc.* 27:409
88. Kniker, W. T. 1970. *Fed. Prod.* 29:287
89. Cochrané, C. G., Hawkins, D. J. 1968. *J. Exp. Med.* 127:137–54
90. Fisher, E. R., Bark, J. 1961. *Am. J. Pathol.* 39:665–79
91. Valdes, A. J., Senterfit, L. B., Pollack, A. D., Germuth, F. G. 1969. *Johns Hopkins Med. J.* 124:9–17
92. Notkins, A. L., Mahar, S., Scheele, C., Goffman, J. 1966. *J. Exp. Med.* 124: 81–97
93. Oldstone, M. B. A., Dixon, F. J. 1967. *Science* 158:1193–95
94. Porter, D. D., Larsen, A. E. 1967. *Proc. Soc. Exp. Biol. Med.* 126:680–82
95. Henson, J. B., Gorham, J. R., Padgett, G. A., Davis, W. C. 1969. *Arch. Pathol.* 87:21–28
96. Hirsch, M. S., Allison, A. C., Harvey, J. J. 1969. *Nature (London)* 223:739–40
97. Oldstone, M. B. A., Dixon, F. J. 1969. *J. Exp. Med.* 129:483–505
98. Porter, D. D., Larsen, A. E., Porter, H. G. 1969. *J. Exp. Med.* 130:575–93
99. Oldstone, M. B. A., Dixon, F. J. 1970. *J. Exp. Med.* 131:1–19
100. Henson, J. B., McGuire, T. C. 1971. *Am. J. Clin. Pathol.* 56:306–14
101. Oldstone, M. B. A., Dixon, F. J. 1971. *J. Immunol.* 106:1260–63
102. Porter, D. D., Porter, H. G. 1971. *J. Immunol.* 106:1264–66
103. Banks, K. L., Henson, J. B., McGuire, T. C. 1972. *Lab. Invest.* 26:701–7
104. Oldstone, M. B. A., Aoki, T., Dixon, F. J. 1972. *Proc. Nat. Acad. Sci.* 69: 134–38
105. Dixon, F. J., Oldstone, M. B. A., Tonietti, G. 1971. *J. Exp. Med.* 134:65s–71s
106. Mellors, R. C., Shirai, T., Aoki, T., Huebner, R. J., Krawczynski, K. 1971. *J. Exp. Med.* 133:113–32
107. Lambert, P. H., Dixon, F. J. 1968. *J. Exp. Med.* 127:507–22
108. Lambert, P. H., Dixon, F. J. 1970. *Clin. Exp. Immunol.* 6:829–39
109. Tonietti, G., Oldstone, M. B. A., Dixon, F. J. 1970. *J. Exp. Med.* 132:89–109
110. Oldstone, M. B. A., Dixon, F. J. 1972. *Science* 175:784–86
111. Heymann, W., Hackel, D. B., Harwood, S., Wilson, S. G. F., Hunter, J. L. P. 1959. *Proc. Soc. Exp. Biol. Med.* 100: 660–64
112. Edgington, T. S., Glassock, R. J., Dixon, F. J. 1967. *Science* 155:1432–34
113. Weigle, W. O., High, G. J. 1967. *J. Immunol.* 98:1105–14
114. Weigle, W. O., Nakamura, R. M. 1969. *Clin. Exp. Immunol.* 4:645–57
115. Gleichmann, H., Gleichmann, E., Andre-Schwartz, J., Schwartz, R. S. 1972. *J. Exp. Med.* 135:516–32
116. Linder, E., Edgington, T. S. 1972. *Fed. Proc.* 31:766
117. Linder, E., Pasternack, A., Edgington, T. S. 1972. *Clin. Immunol. Immunopathol.* 1:104–21
118. Morel-Maroger, L., Leathem, A., Richet, G. 1972. *Am. J. Med.* 53:170–84
119. Gerber, M. A., Paronetto, F. 1971. *Lancet* 1:1097–99
120. Roy, L. P., Westberg, N. G., Michael, A. F. 1973. *Clin. Exp. Immunol.* 13: 553–59
121. Druet, P., Bariety, J., Bernard, D., Lagrue, G. 1970. *Presse Med.* 78:583–87
122. Berger, J., Yaneva, H., Hinglais, N. 1971. *Advan. Nephrol.* 1:11–30
123. Lowance, D. C., Mullins, J. D., McPhaul, J. J. Jr. 1973. *Kidney Int.* 3:167–76
124. Hyman, L. R., Wagnild, J. P., Beirne, G. J., Burkholder, P. M. 1973. *Kidney Int.* 3:397–408
125. Agnello, V., Winchester, R. J., Kunkel, H. G. 1970. *Immunology* 19:909–19
126. Ashe, W. K., Daniels, C. A., Scott, G. S., Notkins, A. L. 1971. *Science* 172: 176–77
127. Winchester, R. J., Kunkel, H. G., Agnello, V. 1971. *J. Exp. Med.* 134:286s–295s
128. Bianco, C., Patrick, R., Nussenzweig, V. 1970. *J. Exp. Med.* 132:702–20
129. Dickler, H. B., Kunkel, H. G. 1972. *J. Exp. Med.* 136:191–96
130. Paraskevas, F., Lee, S-T, Orr, K. B., Israels, L. G. 1972. *J. Immunol.* 108: 1319–27
131. Theofilopoulos, A. N., Bokisch, V. A., Dixon, F. J. 1973. *Fed. Proc.* 32:992
132. Grupe, W. E. 1968. *Pediatrics* 42: 474–82
133. McIntosh, R. M., Kaufman, D. B., Kulvinskas, C., Grossman, B. J. 1970. *J. Lab. Clin. Med.* 75:566–77

134. McIntosh, R. M., Kulvinskas, C., Kaufman, D. B. 1971. *Int. Arch. Allergy* 41:700–15
135. Adam, C., Morel-Maroger, L., Ricket, G. 1973. *Kidney Int.* 3:334–41
136. Iwasaki, Y. et al 1967. *Surg. Gynecol.* Obstet. 124:1–24
137. Joekes, A. M., Gabriel, J. R. T., Goggin, M. J. 1972. *Nephron* 9:162–70
138. Wilson, G. S. 1967. *Hazards of Immunization,* p. 256. New York: Oxford Univ.
139. Kilcoyne, M. M. et al 1972. *Lancet* 1: 17–20
140. Michael, A. F. Jr., Drummond, K. N., Good, R. A., Vernier, R. L. 1966. *J. Clin. Invest.* 45:237–48
141. Dodge, W. F., Spargo, B. H., Bass, J. A., Travis, L. B. 1968. *Medicine* 47: 227–67
142. Fish, A. J., Herdman, R. C., Michael, A. F., Pickering, R. J., Good, R. A. 1970. *Am. J. Med.* 48:28–39
143. Kimmestiel, P., Kim, O. J., Beres, J. 1962. *Am. J. Clin. Pathol.* 38:280–96
144. Andres, G. A., Accinni, L., Hsu, K. C., Zabriskie, J. B., Seegal, B. C. 1966. *J. Exp. Med.* 123:399–412
145. Lewy, J. E., Salinas-Madrigal, L., Herdson, P. B., Pirani, C. L., Metcoff, J. 1971. *Medicine* 50:453–501
146. Jennings, R. B., Earle, D. P. 1961. *J. Clin. Invest.* 40:1525–95
147. Zabriskie, J. B., Utermohlen, V., Read, S. E., Fischetti, V. A. 1973. *Kidney Int.* 3:100–4
148. Zabriskie, J. B. 1971. *J. Exp. Med.* 134:180s–192s
149. Treser, G. et al 1970. *J. Clin. Invest.* 49:762–68
150. Markowitz, A. S., Clasen, R., Nidus, B. D., Ainis, H. 1967. *J. Immunol.* 98: 161–70
151. Rapaport, F. T., Markowitz, A. S., McCluskey, R. T., Hanaoka, T. 1969. *Surgery* 66:34–41
152. Black, J. A., Challacombe, D. N., Ockenden, B. C. 1965. *Lancet* 2:921–24
153. Stickler, G. B. et al 1970. *Am. Heart J.* 79:426–27
154. Kaufman, D. B., McIntosh, R. 1971. *Am. J. Med.* 50:262–68
155. Tu, W. H., Shearn, M. A., Lee, J. C. 1969. *Ann. Intern. Med.* 71:335–41
156. Gutman, R. A., Striker, G. E., Gilliland, B. C., Cutler, R. E. 1972. *Medicine* 51: 1–25
157. Braunstein, G. D., Lewis, E. J., Galvanek, E. G., Hamilton, A., Bell, W. R. 1970. *Am. J. Med.* 48:643–48
158. Bhorade, M. S., Carag, H. B., Lee, H. J., Potter, E. V., Dunea, G. 1971. *J. Am. Med. Assoc.* 216:1159–66
159. Hill, L. L., Singer, D. B., Falletta, J., Stasney, R. 1972. *Pediatrics* 49: 260–66
160. Allison, A. C. et al 1969. *Lancet* 1: 1232–38
161. Ward, P. A., Kibukamusoke, J. W. 1969. *Lancet* 1:283–85
162. Hendrickse, R. G. et al 1972. *Lancet* 1: 1143–48
163. McCoy, R. C., Tisher, C. C. 1972. *Am. J. Pathol.* 68:339–58
164. Wilson, C. B., Dixon, F. J., Evans, A. S., Glassock, R. J. *Clin. Immunol. Immunopathol.* In press
165. Fiala, M. et al 1973. *Nephron* 11:276–86
166. Combes, B. et al 1971. *Lancet* 2:234–37
167. Feizi, T., Gitlin, N. 1969. *Lancet* 2: 873–76
168. Dayan, A. D., Stokes, M. I. 1972. *Brit. Med. J.* 2:374–76
169. Sutherland, J. C., Mardiney, M. R. 1973. *J. Nat. Cancer Inst.* 50:533–644
170. Tennant, F. S. Jr. 1968. *Texas Rep. Biol. Med.* 26:603–12
171. Koffler, D., Agnello, V., Thoburn, R., Kunkel, H. G. 1971. *J. Exp. Med.* 134:169s–179s
172. Agnello, V., Koffler, D., Eisenberg, J. W., Winchester, R. J., Kunkel, H. G. 1971. *J. Exp. Med.* 134:228s–241s
173. Freseo, R. 1968. *Fed. Proc.* 27:246
174. Györkey, F., Sinkovics, J. G., Min, K. W., Györkey, P. 1972. *Am. J. Med.* 53: 148–58
175. Koffler, D., Sandson, J., Kunkel, H. G. 1968. *J. Clin. Invest.* 47:55
176. Naruse, T., Kitomura, K., Miyakawa, Y., Shibata, S. 1973. *J. Immunol.* 110: 1163–66
177. Bariety, J., Druet, P., Laliberte, F., Sapin, C. 1971. *Am. J. Pathol.* 65:293–302
178. Milgrom, F., Klassen, J., Fuji, H. 1971. *J. Exp. Med.* 134:193s–207s
179. Urizar, R. E., Michael, A., Sisson, S., Vernier, R. L. 1968. *Lab. Invest.* 19: 437–50
180. Urizar, R. E., Herdman, R. C. 1970. *Am. J. Clin. Pathol.* 53:258–66
181. Fillastre, J. P., Morel-Maroger, L., Richet, G. 1971. *Lancet* 1:1243–44
182. Glassock, R. J., Feldman, D., Reynolds, E. S., Dammin, G. J., Merrill, J. P. 1968. *Medicine* 47:411–54
183. Edgington, T. S., Lee, S., Dixon, F. J. 1969. *J. Immunol.* 103:528–36

184. Milgrom, F., Litvak, B. I., Kano, K., Witebsky, E. 1966. *J. Am. Med. Assoc.* 198:226–30
185. Iwaski, Y., Talmage, D., Starzl, T. E. 1967. *Transplantation* 5:191–206
186. Gotoff, S. P., Fellers, F. X., Vawter, G. F., Janeway, C. A., Rosen, F. S. 1965. *N. Engl. J. Med.* 273:524–29
187. West, C. D., McAdams, A. J., McConville, J. M., Davis, N. C., Holland, N. H. 1965. *J. Pediat.* 67:1089–1112
188. Herdman, R. C. et al 1970. *Medicine* 49:207–26
189. Gewurz, H., Pickering, R. J., Mergenhagen, S. E., Good, R. A. 1968. *Int. Arch. Allergy* 34:556–70
190. Kohler, P. F., Ten Bensel, R. 1969. *Clin. Exp. Immunol.* 4:191–202
191. Lewis, E. J., Carpenter, C. B., Schur, P. H. 1971. *Ann. Intern. Med.* 75:555–60
192. McLean, R. H., Michael, A. F. 1973. *J. Clin. Invest.* 52:634–44
193. Westberg, N. G., Noff, G. B., Boyer, J. T., Michael, A. F. 1971. *J. Clin. Invest.* 50:642–49
194. Vallota, E. G., Forristal, J., Spitzer, R. E., Davis, N. C., West, C. D. 1970. *J. Exp. Med.* 131:1306–24
195. West, C. D., Ruley, E. J., Forristal, J., Davis, N. C. 1973. *Kidney Int.* 3: 116–25
196. Verroust, P. J., Wilson, C. B., Dixon, F. J. Submitted to *Kidney Int.*
197. Verroust, P. J., Wilson, C. B., Cooper, N. R., Edgington, T. S., Dixon, F. J. *J. Clin. Invest.* In press

Copyright 1974. All rights reserved

DIPHENYLHYDANTOIN AS AN ANTIARRHYTHMIC DRUG

❖7098

Arthur J. Atkinson Jr., M.D. and Richard Davison, M.D.
The Clinical Pharmacology Laboratory and Intensive Care Area, Division of Medicine, Northwestern Memorial Hospital, and the Departments of Medicine and Pharmacology, Northwestern University Medical School, Chicago, Illinois

INTRODUCTION

Antiarrhythmic agents in current use suffer from disadvantages of less than complete efficacy, inconvenient administration, and often troublesome toxicity. Initial reports of the successful treatment of cardiac arrhythmias with diphenylhydantoin (DPH) were received with particular enthusiasm because this drug is well-absorbed orally, has a long duration of action, and has a proven record of relative safety in the long-term treatment of patients with epilepsy. Although this enthusiasm carried over into subsequent reviews of antiarrhythmic therapy with DPH (1, 2), even now DPH continues to be used relatively infrequently for this purpose. The reasons for this lack of popularity are to be found in the limited inherent efficacy of DPH, in the toxicity resulting from intravenous injection of DPH, and in difficulties surrounding the proper administration of this drug that tend to exaggerate its apparent ineffectiveness as well as its toxicity. A consideration of these topics forms the basis of this review.

ANTIARRHYTHMIC EFFICACY OF DPH

Harris & Kokernot first reported in 1950 that DPH was effective in the treatment of ectopic ventricular tachycardia produced in dogs by the occlusion of the anterior descending branch of the left coronary artery (3). This use of DPH was suggested by apparent similarities in the origin of cardiac arrhythmias following coronary occlusion and focal epileptogenic discharges. Seven years later Leonard reported the successful clinical use of DPH in treating a patient with an inferior wall myocardial infarction and ventricular tachycardia (4). This arrhythmia had previously been refractory to procainamide therapy, even though procainamide had been given in a dose sufficient to produce hypotension and widening of the QRS complex. The antiarrhythmic efficacy of DPH has since been well documented in a number of clinical settings, although success has not been uniform (Table 1) (1, 5–9).

Table 1 Efficacy of DPH antiarrhythmic therapy[a]

Clinical Setting	Digitalis Therapy				Ischemic Heart Disease					Other Heart Disease		
Arrhythmia	Ref. 5[b]	Ref. 6	Ref. 1	Ref. 7	Ref. 6	Ref. 1	Ref. 7	Ref. 8	Ref. 9	Ref. 6	Ref. 7	Ref. 8
Supraventricular:												
Atrial fibrillation					1/1		0/3	4/1		0/1		1/6
Atrial flutter					0/1		0/2			0/1	0/1	1/0
PAT[c]	2/0	1/0		8/0						1/0	4/5	0/1
PAT with block	1/0	2/0										
Nodal tachycardia	2/2		0/2	0/1							1/0	
Unspecified or other	14/15	1/0		0/1			0/2	2/2		0/1	0/4	2/2
Ventricular:												
VPBs[d]	8/0	2/0		4/0			0/1	5/0		1/0	0/3	1/0
VPBs with atrial fibrillation		1/0		2/2								
Bigeminy	4/0	1/0		5/0	2/1		1/1	2/0		1/0	1/0	0/1
Ventricular tachycardia					1/0			5/0	0/10		1/10	
Ventricular fibrillation												1/0
Unspecified			29/18			134/84	0/1					

[a] Shown as patients successfully treated/patients unsuccessfully treated.

[b] Arrhythmias not necessarily due to digitalis.

[c] PAT — paroxysmal atrial tachycardia.

[d] VPBs — ventricular premature beats.

Treatment of Digitalis Toxicity

The ability of DPH to abolish ectopic ventricular tachycardia following experimental myocardial infarction prompted Mosey & Tyler to evaluate the effectiveness of DPH in treating ouabain-induced ventricular tachycardia in dogs (10). Single DPH doses of 10–30 mg/kg were able to revert this arrhythmia to normal sinus rhythm for periods of 20 min to more than 3 hr. DPH also appeared to reverse the emesis and lethargy that accompanied the infusion of cardiotoxic doses of ouabain. Subsequent studies by Helfant et al demonstrated that the initial treatment with 5 mg/kg DPH increased by 72–224% the dose of acetylstrophanthidin necessary to produce ventricular tachycardia in dogs (11). They made the additional important observation that DPH in these doses did not alter the inotropic response to acetylstrophanthidin. In fact, continued enhancement of the rate of rise of left ventricular pressure (dp/dt) was obtained with acetylstrophanthidin doses that would have been cardiotoxic had DPH prophylactic therapy not been given.

So far no clinical studies have been reported in which DPH therapy has been used to extend the nontoxic range of digitalis therapy. However, the usefulness of DPH in the treatment of digitalis-induced arrhythmias has been amply confirmed (Table 1). Indeed, the primary indication for the use of DPH as an antiarrhythmic drug is in the treatment of arrhythmias due to digitalis (12).

Treatment of Arrhythmias Unrelated to Digitalis in Patients with Heart Disease

In other clinical settings DPH has been less uniformly successful as an antiarrhythmic drug. In Table 1 are shown results of DPH therapy of patients with ischemic heart disease and heart disease due to other causes. In the treatment of atrial arrhythmias, DPH efficacy appears to be 50% or less. In most reports the response of patients with ventricular arrhythmias is slightly better, but far from consistent. Thus, in a particularly well-conducted study by Stone et al, none of 10 patients with recurrent ventricular tachycardia due to ischemic heart disease responded to DPH therapy (9).

Particular interest has centered on the long-term prevention of cardiac arrhythmias in survivors of myocardial infarction. It has been proposed that such arrhythmias are an important cause of late sudden death in these patients and that chronic antiarrhythmic therapy might significantly improve their survival (13). One controlled trial has been conducted in which DPH therapy in standard doses of 300 or 400 mg/day did not improve prognosis for patients in the first year following infarction (14).

Treatment of Arrhythmias During Surgery or Cardiac Catheterization

DPH has been used to treat potentially serious arrhythmias associated with anesthesia, cardiac surgery, and cardiac catheterization. Evaluation of these results is complicated by the fact that these arrhythmias are frequently self-limited and the factors precipitating them are often of short duration. Nonetheless DPH appears to be generally effective in these settings.

In one report DPH gave excellent results in 82% of arrhythmias associated with anesthesia (1), and in another DPH was found effective in preventing cyclopropane-associated arrhythmias as well as in treating ventricular arrhythmias arising during general anesthesia with other agents (15). DPH also gave excellent results in treating arrhythmias in 137 of 226 patients during and after cardiac surgery (1). In a smaller series of 14 patients with arrrhythmias following cardiac surgery, DPH was uniformly effective in treating ventricular extrasystoles, bigeminy, and nodal tachycardia, but only controlled 1 of 2 patients with recurrent ventricular tachycardia (16). Only 2 of these 14 patients were receiving digitalis and this drug was not thought to have caused these arrhythmias, although it has been shown that myocardial sensitivity to the toxic effects of digitalis s markedly increased in the first 24 hr following cardiopulmonary bypass (17).

In arrhythmias associated with cardiac catheterization, an excellent response to DPH therapy was reported in 79% of 132 patients (1).

MECHANISM OF DPH ANTIARRHYTHMIC ACTION

Proposed mechanisms for the antiarrhythmic effects of DPH therapy have emphasized both indirect and direct cardiac actions of this drug. At the present time, it would be hard to choose one mechanism as being exclusively responsible for antiarrhythmic efficacy.

Indirect Cardiac Actions

Coronary vasodilation has been suggested by Gupta et al as a possible mechanism for DPH antiarrhythmic action (18). These workers used a dog heart-lung preparation and found that DPH doses of 5–20 mg/kg caused an increase in coronary blood flow when coronary perfusion pressure was kept constant. However, even if coronary blood flow increases when DPH is given to patients in somewhat lower doses, there is no convincing evidence that this would have antiarrhythmic efficacy (1).

Standaert and colleagues have emphasized that neurodepression may be a major mechanism of DPH antiarrhythmic action (19–21). Thus, Raines et al showed that DPH reversed ouabain-induced ventricular tachycardia in cats with high, but not low, adrenergic activity (20). Subsequently, Gillis et al demonstrated that DPH in doses as low as 2 mg/kg reduced sympathetic activity (21). Since increased sympathetic tone plays an important role in the genesis of arrhythmias following experimental myocardial infarction (22–24) and during surgery (25), as well as arrhythmias associated with digitalis toxicity, this action of DPH is likely to be significant. DPH also depresses vagal and phrenic nerve activity (21). The mechanism of DPH neurodepression is presumably the same membrane-stabilizing effect that accounts for its usefulness in treating seizure disorders. These effects have been attributed to a DPH-induced increase in the extrusion of sodium from nerve cells (26).

Direct Cardiac Actions

The direct cardiac actions of DPH, in concentrations that are clinically therapeutic, have been studied recently in isolated Purkinje fibers and in atrial tissue. DPH reduced Purkinje fiber automaticity by decreasing the slope of spontaneous phase-4 depolarization, as well as by increasing the maximum diastolic potential reached before the beginning of spontaneous phase-4 depolarization (27). DPH reduced action potential duration to a greater extent than the effective refractory period, thus tending to decrease the likelihood of initiating sustained re-entrant arrhythmias. In acutely depressed Purkinje fibers DPH improved membrane responsiveness, defined as the relationship of the rate of rise of the action potential during phase 0 to the membrane potential at the instant of fiber activation. Because responsiveness is a determinant of conduction velocity, this action might also be important in terminating re-entrant arrhythmias. Strauss et al showed that DPH also reduces the slope of phase-4 depolarization in sinus node tissue, but only at concentrations 100-fold higher than required to depress Purkinje fiber automaticity (28). This may provide one possible explanation for the greater success of DPH in treating ventricular arrhythmias as opposed to arrhythmias due to increased atrial automaticity. Although DPH did not affect action potential duration or the effective refractory period of atrial tissue, membrane responsiveness was improved. Under certain conditions conduction velocity could increase as a result of such action and atrial arrhythmias having a re-entrant mechanism might terminate. Since re-entry has been demonstrated in some patients with paroxysmal supraventricular tachycardia (29), the success of DPH in treating this arrhythmia is consistent with such a mechanism of action.

Subsequent investigators have noted that the cardiac tissue used in these initial studies was bathed in Tyrode's solution containing only 3 m*M* potassium, whereas the physiological concentration range is 5.0–5.5 m*M* (30, 31). Experiments made using these higher concentrations of potassium in the bathing solution indicate that DPH decreases rather than increases membrane responsiveness. However, in clinical studies DPH has little or no effect on intraventricular conduction, in contrast to procainamide, which markedly decreases membrane responsiveness and can prolong intraventricular conduction time (32). Caution in the extrapolation of these in vitro results to the in vivo situation is further warranted by the recent finding that conduction is more rapid in Purkinje fibers perfused with blood rather than Tyrode's solution (33). Fiber automaticity in both perfusates was comparable.

Little is known about the ionic basis for the direct cardiac actions of DPH. It has been proposed that DPH reduces ectopic pacemaker automaticity by increasing potassium conductance during phase 4, thus preventing spontaneous diastolic depolarization (34). An action of DPH on myocardial sodium-potassium adenosine triphosphatase does not appear to be involved since DPH abolishes ouabain-induced toxic arrhythmias without reversing ouabain inhibition of this enzyme (35).

DPH ADMINISTRATION

Despite clear-cut evidence that DPH is effective in treating certain cardiac arrhythmias in some patients, the clinical use of this drug has met with only partial success. Very likely, some failures of DPH therapy result from administration of a dose too low to provide therapeutic blood levels. Indeed, reports including blood level data are the exception rather than the rule.

Bigger et al were the first to point out that 90% of arrhythmias responsive to DPH were abolished at plasma levels less than 18 μg/ml; three quarters responding within the blood level range of 10–18 μg/ml (36). Therapeutic success was limited to patients with supraventricular tachycardia or ventricular premature systoles and effective plasma levels ranged from 4–24 μg/ml. A critically effective plasma level could be demonstrated in many patients, below which the arrhythmia would return. Since most neurotoxicity also correlates with the DPH plasma level, effective and safe antiarrhythmic therapy with this drug depends on the plasma level provided by the selected therapeutic program. Recent progress has been made in delineating three important phases of DPH therapy: initial therapy, "dilantinization," and maintenance therapy.

Initial Therapy

In many clinical situations, rapid arrhythmia control is essential and DPH must be given by intravenous injection. However, the rate of injection should not exceed 50 mg/min. Because plasma DPH levels can not be measured rapidly enough to monitor initial therapy, Bigger et al have recommended using repeated intravenous injections of 100 mg DPH at 5-min intervals (37). When given in this way, plasma levels (Y) 5 min after the preceeding dose can be estimated from the cumulative dose (X), in mg/kg, from the formula $Y = 2.64 + 2.22\,X$, the standard deviation of Y being ± 3.3 μg/ml. Injections should be repeated until the arrhythmia is abolished or until an estimated plasma level of 18 μg/ml is exceeded or toxicty supervenes.

Dilantinization

Once an effective initial plasma level has been reached, supplemental doses of DPH need to be given over the next two days before maintenance therapy can be started. The term "dilantinization," akin to digitalization, has been coined to describe this process (38). Usually a total of 1000 mg DPH is given over the first 24 hr of therapy, followed by 500 mg the next day to complete dilantinization. This should be given either orally or by intravenous injection, since DPH is absorbed slowly from intramuscular injection sites and precipitates if added to intravenous infusions of fluids that have a low pH (39, 40). The additional DPH is needed to maintain plasma levels in the therapeutic range not only because of drug losses due to metabolism but predominantly because drug distribution to slowly equilibrating tissues is occurring. Suzuki et al (41) have studied these processes with a two-compartment pharmacokinetic model (Figure 1) that gives results agreeing closely with the initial DPH level predictions of Bigger and co-workers (37).

Since the implications of these pharmacokinetic studies are not intuitively obvious, the SAAM digital computer program of Berman & Weiss (42) has been used to simulate the plasma levels that would be expected if subject Y of Suzuki et al were treated by the method of Bigger et al (Figure 2). Assuming that his arrhythmia would be abolished when plasma levels exceed 10 μg/ml, initial arrhythmia control would require two 100-mg injections. After 18.5 min the arrhythmia would recur as plasma levels fell below 10 μg/ml. A third 100-mg injection of DPH at 20 min

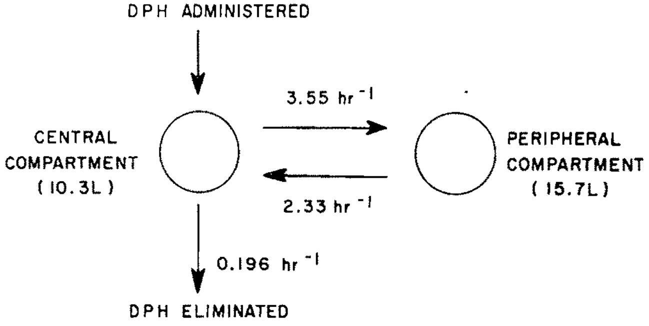


Figure 1 Model proposed by Suzuki et al for the multicompartmental distribution of DPH in subject *Y*, weighing 57 kg (41).

would again abolish the arrhythmia for another 70 min. Cumulative DPH elimination is also shown in Figure 2 to emphasize that drug distribution to the peripheral compartment is the major reason that supplemental DPH is required so soon after initial arrhythmia control, since only 34.5 mg or 12% of the administered DPH would have been eliminated during the first 90 min of therapy. Failure to appreciate the importance of this phenomenon may lead to a premature conclusion that DPH therapy has been ineffective.

Recent developments have made possible determination of DPH plasma levels in less than an hour, so that they can be used to guide therapy during the period of dilantinization (43). Even during initial therapy, when the arrhythmia is abolished or recurs, a blood specimen can be obtained for later reference in establishing the plasma level range that will be needed for arrhythmia control. Although such a therapeutic approach can be expected to improve the overall results of antiarrhythmic treatment with DPH, even at plasma levels above 10 μg/ml 9 of the 10 patients with recurrent ventricular tachycardia reported by Stone et al failed to respond to DPH therapy (9).

Maintenance Therapy

After dilantinization is complete, maintenance doses are needed to replace metabolized DPH so that plasma levels remain in the therapeutic range. Proper dose selection is complicated by the fact that the relationship between steady state plasma level and dose is not linear. On the average, a daily DPH dose of 300 mg gives a plasma level of 10 μg/ml and a dose of 400 mg doubles that level to 20 μg/ml (38). The main route of DPH metabolism involves hydroxylation of one of the phenyl rings followed by glucuronide conjugation (44, 45). The initial parahydroxylation step has been shown to be rate-limiting in studies with rat liver microsomes (46), and Remmer was the first to suggest that the nonlinear relationship between DPH dose and plasma level might be due to saturation of this enzymatic step (47). In support of this explanation, limited studies by Gerber & Wagner (48) and by

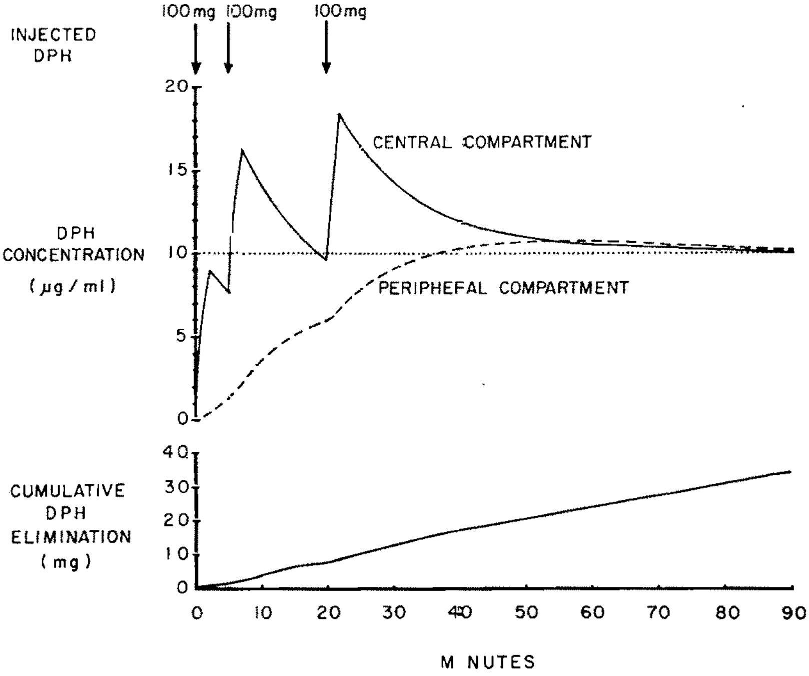


Figure 2 Simulated DPH therapy of a cardiac arrhythmia in subject *Y* based on the model shown in Figure 1. The arrhythmia is abolished when plasma DPH levels exceed 10 μg/ml (dotted line). DPH is injected in 100-mg doses at 0, 5, and 20 min (arrows). DPH concentration in the central compartment, which includes plasma, is shown by the solid line and in the peripheral compartment by the broken line in the upper panel. Cumulative DPH elimination is shown in the lower panel.

Atkinson & Shaw (49) have shown that in patients the elimination of DPH appears to follow Michaelis Menten kinetics and that the Michaelis constant for the rate-limiting step is of the same order of magnitude as that found for the parahydroxylation of DPH in a rat liver microsomal preparation (50).

However, the response of individual patients to standardized doses of DPH is too variable to base dose selection on pharmacokinetic predictions alone. Thus, in patients treated with 300 mg DPH daily, plasma levels ranged from 1–53 μg/ml (51). Therefore, plasma level monitoring is essential for successful long-term antiarrhythmic therapy with DPH. In this regard, it should be noted that DPH levels were not routinely monitored in the large collaborative trial that failed to show a decrease in mortality in survivors of myocardial infarction treated with prophylactic DPH (14). However, in one participating hospital such measurements were made during the study year, and it was found that there were 10 deaths in the 64 patients whose levels averaged less than 10 μg/ml, and no deaths in the 32 patients whose levels exceeded 10 μg/ml.

The sources of variation in patients' response to DPH include compliance with the prescribed regimen, decreased parahydroxylating capacity due to liver disease or a genetic deficiency, and interactions with other drugs affecting the metabolism of DPH (52, 53). In outpatient practice, lack of compliance is a particularly common cause of inadequate seizure control (52) and is likely to be an important consideration in long-term antiarrhythmic therapy as well. To the extent that compliance is related to convenience, this can be improved by administering DPH in a single daily dose rather than in divided doses. Buchanan et al recently have shown that comparable levels are obtained by either method of administration (54).

Special caution is needed in the treatment of uremic patients with DPH. Odar-Cederlöf et al reported that protein binding is impaired in these patients so that therapeutically effective total DPH levels are lower than in nonuremic patients (55). Probably toxicity will also occur at lower than usual DPH levels. Reidenberg et al have shown that a qualitative change in drug-binding proteins is the most likely cause for this increase in the unbound fraction of DPH, which ranged from 8–25% in uremic patients compared to a normal value of 7.4% (56).

DPH TOXICITY

Initial enthusiasm for antiarrhythmic therapy with DPH has been tempered by reports of serious cardiovascular toxicity following its intravenous administration. In addition, long-term antiarrhythmic therapy with this drug can be expected to pose all of the same problems as chronic anticonvulsant therapy.

Intravenous DPH Therapy

Intravenous administration of DPH is hazardous. Even with precautionary electrocardiographic monitoring, fatalities from respiratory arrest, arrhythmias, and hypotension have occurred. Clinical data from reports of cardiac arrest after intravenous injection of DPH are summarized in Table 2 (7, 57–61). Apnea preceded signs of cardiac toxicity in two reported fatalities from intravenous DPH therapy (7, 57). Respiratory arrest followed by cardiac asystole also was the cause of death in two dogs described in Harris & Kokernot's original investigation of the antiarrhythmic properties of DPH (3). These fatalities in dogs resulted from excessively rapid injection of DPH. Thus, rapid injection of as little as 20 mg/kg caused respiratory arrest, while slow infusion of 50 mg/kg was well tolerated.

In other experiments in dogs, infusion of DPH in lethal doses has produced a nodal or slow idioventricular rhythm followed by asystole (62). Depression of cardiac automaticity has likewise been noted in most of the reports of severe cardiac toxicity following intravenous DPH therapy. However, ventricular fibrillation was described in one of the patients shown in Table 2 (59), and was reported in another case by Mercer & Osborne (1). All patients in Table 2 were over 65 years old, were being treated with digitalis for congestive heart failure, and received DPH at a faster rate of infusion than recommended by Bigger et al (37). Rosen et al reported a nonfatal episode of severe DPH cardiac toxicity in a patient who had a 6-sec period of sinus arrest that responded to a single blow on the chest (5), and Eddy & Singh described a patient with artrial fibrillation and myocardial infarction who was

Table 2 Cardiac arrest after intravenous DPH

Source	Age	Digitalis	CHF	Indication for DPH	DPH Dose and Rate	Clinical Course
Ref. 7	67	+	+	? Recurrent pulmonary embolism	250 mg in 1 min	Hypotension → apnea ECG not recorded
Ref. 57	67	+	+	? Digitalis toxicity	250 mg in 5 min	Apnea → atrioventricular block with idioventricular rhythm → asystole
Ref. 58 Case 1	67	+	+	Atrial flutter	250 mg in 2 min	Sinus rhythm → sinus arrhythmia → idioventricular rhythm → asystole
Ref. 58 Case 2	70	+	+	Atrial flutter	250 mg in 3 min	Increased atrioventricular block → asystole
Ref. 59	85	+	+	Supraventricular tachycardia, ? Digitalis toxicity	150 mg in 3 min	Ventricular fibrillation
Ref. 60	82	+	+	Supraventricular tachycardia	250 mg in 2½ min	Atrioventricular nodal rhythm → asystole
Ref. 61	76	+	+	? Digitalis toxicity	250 mg in 3 min	Atrioventricular nodal rhythm → idioventricular rhythm → asystole

treated with DPH and then developed bradycardia followed by an atrioventricular nodal rhythm that required transvenous pacing (8). Transient sinoatrial arrest requiring temporary pacemaker insertion also was noted in a 57-year-old patient with myocardial infarction whose frequent ventricular extrasystoles were treated with DPH and lidocaine (63). Possibly in this instance lidocaine contributed to the depression of the sinus node (63, 64). Although such reactions are usually associated with intravenous DPH therapy, idioventricular rhythm and sinus bradycardia have occurred even after oral DPH therapy (65).

Even though atrioventricular block and bradycardia have been mentioned as toxic effects of DPH (6), much of the rationale for using this drug in preference to other antiarrhythmic agents in treating patients with digitalis toxicity lies in the fact that DPH usually enhances rather than depresses atrioventricular conduction. This has been shown in experimental studies in dogs (66) as well as in patients (32). But this enhancement of atrioventricular conduction has been hazardous in treating patients with atrial flutter because occasionally 1:1 conduction has been precipitated (67, 68). This possible complication, together with the general ineffectiveness of DPH in treating atrial fibrillation and flutter, should mitigate against the use of this drug in these conditions.

Hypotension accompanying intravenous injection of DPH was noted in the classic animal studies of DPH efficacy (3). Harris & Kokernot also pointed out that the magnitude of hypotension was related to the speed of injection. Hypotension has been cited in most clinical reports of antiarrhythmic therapy with DPH and has been reported to occur with an incidence ranging from 2–8% (5–8, 15). The severity of reported hypotension varies considerably, from the fatal case reported by Karliner (7) to the fall of 10–20 mm Hg described by Eddy & Singh (8). In clinical reports the fall in blood pressure also correlates with the dose and speed of DPH injection. When given by the incremental dose method recommended by Bigger et al little change in blood pressure occurred until a total DPH dose of 500 mg had been injected. At higher doses there was a 10–30 mm Hg fall in systolic blood pressure (36).

In addition to bradyarrhythmias resulting from the effects of DPH on cardiac automaticity, this drug appears to lower blood pressure by decreasing the force of myocardial contraction and by causing peripheral vasodilation. These actions of DPH were studied by Lieberson et al who injected 250 mg DPH intravenously over 5 min in 9 patients with heart disease and found a maximum fall in blood pressure of 10 mm Hg (69). All patients were receiving digitalis therapy for congestive heart failure, but none were in congestive heart failure at the time of the study. Depression of myocardial contractile force was indicated by an average rise in left ventricular end-diastolic pressure of 10 mm Hg and an average 12% fall in the maximum rate of rise of left ventricular pressure, but these values returned to normal within 25 min. The average cardiac index was unchanged, suggesting that peripheral vasodilation counteracted the effects of depressed myocardial function on cardiac output, but was not more marked. Possibly more severe changes would have been seen had more seriously ill patients been studied.

More detailed investigation of DPH-induced decreases in myocardial contractility and peripheral vascular resistance has been possible in experimental studies with dogs (70, 71). Thus, denervation studies have shown that DPH lowers peripheral resistance by acting directly on peripheral blood vessels (70). Also, by separating the cardiopulmonary and systemic circulations, it has been possible to demonstrate directly that myocardial depression is of greater significance than peripheral vasodilation in DPH-induced hypotension (70). Studies on the interaction of DPH and digitalis have shown that digoxin antagonizes the negative inotropic and peripheral vasodilating actions of DPH (62). It is possible that this interaction explains in part the minor degree of hypotension encountered in the study of digitalized patients by Lieberson et al (69).

Although these toxic effects have generally been ascribed to DPH, Louis et al have pointed out that the diluent usually supplied with DPH is not pharmacologically inert (72). This diluent has a pH of 11 and contains 40% propylene glycol, 10% ethyl alcohol, and water. Although injections of 0.01 *N* NaOH and 10% ethyl alcohol were without effect, cats injected with 40% propylene glycol, or with the diluent supplied by the manufacturer, demonstrated hypotension, transient apnea, and depression of sinoatrial rhythm with emergence of lower pacemakers. Only transient hypotension and a slight bradycardia were observed when 10–25 mg/kg DPH was injected in 0.01 *N* NaOH. Thus, some of the adverse effects following intravenous injection of DPH may have been due to propylene glycol rather than DPH. Although diluent controls were not included in work prior to this report, Scriabine et al have since shown that DPH in lethal doses of 127 ± 19 mg/kg caused major hypotension and cardiac asystole that were not observed when control injections of an ethyl alcohol-saline diluent were made (62).

One toxic effect probably related to the high pH of the diluent is local irritation at the injection site (12). Leonard, in his original clinical report (4), and Rosen et al (5) describe transient burning at the site of intravenous injection. Mercer & Osborne encountered local venous thrombosis unless the vein was flushed after each injection, or the injection was made via an intravenous catheter inserted into a large vein (1). Seuffert et al report diluting DPH to a concentration of 10 mg/ml with lactated Ringer's solution in order to avoid local irritation (15), and Bigger et al followed each dose of DPH with an injection of sterile saline to prevent pain and inflammation at the injection site (37). Unless similar precautions are taken, local pain and venous thrombosis will occur even with injections of newer, premixed DPH solutions.

DPH Toxicity After Initial Therapy

Nausea, dizziness, blurring of vision, tremor, and ataxia were all described as toxic effects in Merritt & Putnam's initial clinical report of DPH therapy in epilepsy (73). Their patients were started on maintenance doses of DPH and these symptoms tended to occur between the third and the tenth day of therapy. However, these toxic reactions may occur on the first day of therapy when loading doses are given.

These symptoms can generally be related to plasma level in patients receiving long-term DPH therapy. Thus, nystagmus on far lateral gaze usually is present at levels above 15 to 25 μg/ml, ataxia at levels above 30 μg/ml, and mental slowing at levels above 40 μg/ml (74). However, in patients treated with DPH loading doses, Stone et al detected neurotoxicity at levels as low as 8.6 μg/ml (9). In our experience also, patients may develop nystagmus, blurred vision, light headedness, and ataxia at DPH levels below 10 μg/ml. Since these symptoms subside in several days without any change in blood level, it appears that some tolerance to DPH neurotoxicity develops. DPH-activated seizures (75) and pseudodegenerative cerebral reactions (76) have been reported in patients with DPH levels above 30 μg/ml.

Common toxic reactions to DPH that are not dose-related have been reviewed recently and include gingival hyperplasia, peripheral neuropathy, and allergic reactions (77, 78), as well as a megaloblastic anemia, which is associated with low serum folate levels and responds to folic acid therapy (78, 79). These reviews also deal with less common but often diagnostically confusing complications of DPH therapy that are outside the scope of this presentation.

CONCLUSIONS

DPH has a limited role as an antiarrhythmic drug. It has been generally ineffective in treating patients with atrial flutter and fibrillation, and untoward reactions have resulted from its enhancement of atrioventricular conduction. The reported fatalities emphasize the marked depressant effects of DPH on sinoatrial rhythmicity, and the use of this drug is probably contraindicated in patients with bradycardia or sick sinus syndrome. Caution is warranted in treating patients over 65 years of age, as most fatal cases have occurred in this age group when DPH dose and rate of administration have exceeded recommended guidelines. High degree atrioventricular block has been cited as a contraindication to DPH therapy, but the usual action of DPH is to enhance atrioventricular conduction so this risk appears to be less for DPH than for quinidine, procainamide, or propranolol. Nevertheless, it might be wise to be prepared for transvenous pacing if the use of DPH is contemplated in such patients.

In contrast to other atrial arrhythmias, paroxysmal atrial tachycardia in patients receiving digitalis has been effectively treated with DPH. DPH appears to be particularly useful in treating ventricular arrhythmias due to digitalis toxicity, but the greater safety of lidocaine makes it perhaps the drug of first choice. Lidocaine is probably also the preferred drug in the emergency treatment of ventricular arrhythmias resulting from other causes. Although the possibility of once daily administration makes DPH an attractive choice for long-term therapy of such arrhythmias, studies with appropriate blood level monitoring are needed to confirm its efficacy.

Thus, at present, DPH appears to be a second-line antiarrhythmic drug that is used most often when other drugs have been tried and found ineffective. Patients in this setting are apt to be critically ill, and particular care in the administration of DPH is essential if serious toxicity is to be avoided.

ACKNOWLEDGMENT

Dr. Atkinson is supported by a Burroughs Wellcome Scholar Award in Clinical Pharmacology.

Literature Cited

1. Mercer, E. N., Osborne, J. A. 1967. *Ann. Intern. Med.* 67:1084–107
2. Damato, A. N. 1969. *Progr. Cardiov. Dis.* 12:1–15
3. Harris, A. S., Kokernot, R. H. 1950. *Am. J. Physiol.* 163:505–16
4. Leonard, W. A. Jr. 1958. *Arch. Intern. Med.* 101:714–17
5. Rosen, M., Lisak, R., Rubin, I. L. 1967. *Am. J. Cardiol.* 20:674–78
6. Conn, R. D. 1965. *N. Engl. J. Med.* 272: 277–82
7. Karliner, J. S. 1967. *Dis. Chest* 51: 256–69
8. Eddy, J. D., Singh, S. P. 1969. *Brit. Med. J.* 4:270–73
9. Stone, N., Klein, M. D., Lown, B. 1971. *Circulation* 43:420–27
10. Mosey, L., Tyler, M. D. 1954. *Circulation* 10:65–70
11. Helfant, R. H., Scherlag, B. J., Damato, A. N. 1967. *Circulation* 36:119–24
12. Conn, R. D. 1967. *Med. Clin. N. Am.* 51:1223–40
13. Denborough, M. A., Lovell, R. R. H., Nestel, P. J., Goble, A. J. 1968. *Lancet* 1:386–88
14. Lovell, R. R. H. et al 1971. *Lancet* 2: 1055–57
15. Seuffert, G. W., Helfant, R. H., Dana, J. F., Urbach, K. F. 1968. *Anesth. Analg. (Cleveland)* 47:334–39
16. Gautam, H. P. 1969. *Brit. Heart J.* 31: 641–44
17. Morrison, J., Killip, T. 1973. *Circulation* 47:341–52
18. Gupta, D. N., Unal, M. O., Bashour, F. A., Webb, W. R. 1967. *Dis. Chest* 51: 248–55
19. Standaert, F. G., Levitt, B., Roberts, J., Raines, A. 1969. *Eur. J. Pharmacol.* 6: 209–16
20. Raines, A., Levitt, B., Standaert, F. G., Sohn, Y. J. 1970. *Eur. J. Pharmacol.* 11: 293–97
21. Gillis, R. A., McClellan, J. R., Sauer, T. S., Standaert, F. G. 1971. *J. Pharmacol. Exp. Ther.* 173:599–610
22. Ebert, P. A., Allgod, R. J., Sabiston, D. C. 1968. *Ann. Surg.* 168:728–35
23. Schaal, S. F., Wallace, A. G., Sealy, W. C. 1969. *Cardiovasc. Res.* 3:241–44
24. Gillis, R. A. 1971. *Am. Heart J.* 81: 677–84
25. Erlanger, H. 1962. *Am. J. Med. Sci.* 243:651–78
26. Pincus, J. H., Rawson, M. D. 1969. *Neurology* 19:419–22
27. Bigger, J. T. Jr., Bassett, A. L., Hoffman, B. F. 1968. *Circ. Res.* 22:221–36
28. Strauss, H. C., Bigger, J. T. Jr., Bassett, A. L., Hoffman, B. F. 1968. *Circ. Res.* 23:463–77
29. Bigger, J. T. Jr., Goldreyer, B. N. 1971. *Circulation* 43:15–26
30. Singh, B. N. 1971. *Brit. J. Pharmacol.* 41:385P–386P
31. Jensen, R. A., Katzung, B. G. 1970. *Circ. Res.* 26:17–27
32. Damato, A. N., Berkowitz, W. D., Patton, R. D., Lau, S. H. 1970. *Am. Heart J.* 79:51–56
33. Rosen, M. R., Gelband, H., Hoffman, B. F. 1972. *Circ. Res.* 30:575–87
34. Bigger, J. T. Jr. 1972. *Hosp. Prac.* 7:69–80
35. Spain, R. C., Chidsey, C. A. 1971. *J. Pharmacol. Exp. Ther.* 179:594–98
36. Bigger, J. T. Jr., Schmidt, D. H., Kutt, H. 1968. *Circulation* 38:363–74
37. Bigger, J. T. Jr., Schmidt, D. H., Kutt, H. 1969. *Am. Heart J.* 77:572–73
38. Kutt, H., McDowell, F. 1968. *J. Am Med. Assoc.* 203:969–72
39. Serrano, E. E., Roye, D. B., Hammer, R. H., Wilder, B. J. 1973. *Neurology* 23: 311–17
40. Wilensky, A. J., Lowden, J. A. 1973. *Neurology* 23:318–24
41. Suzuki, T., Saitoh, Y., Nishihara, K. 1970. *Chem. Pharm. Bull.* 18:405–11
42. Berman, M., Weiss, M. F. 1967. *SAAM Manual.* Washington DC: GPO (PHS Publ. No. 1703)
43. MacGee, J. 1970. *Anal. Chem.* 42: 421–22
44. Butler, T. C. 1957. *J. Pharmacol. Exp. Ther.* 119:1–11
45. Maynert, E. W. 1960. *J. Pharmacol. Exp. Ther.* 130:275–84
46. Gerber, N. et al 1971. *J. Pharmacol. Exp. Ther.* 178:567–79
47. Remmer, H., Hirschmann, J., Greiner, I. 1969. *Deut. Med. Wochenschr.* 94: 1265–72
48. Gerber, N., Wagner, J. G. 1972. *Res. Commun. Chem. Pathol. Pharmacol.* 3: 455–66
49. Atkinson, A. J. Jr., Shaw, J. M. 1973. *Clin. Pharmacol. Ther.* 14:521–28
50. Kutt, H., Verebely, K. 1970. *Biochem. Pharmacol.* 19:675–86
51. Atkinson, A. J. Jr. *Med. Clin. N. Am.* In press
52. Gibberd, F. B., Dunne, J. F., Handley, A. J., Hazelman, B. L. 1970. *Brit. Med. J.* 1:147–49

53. Kutt, H. 1971. *Ann. NY Acad. Sci.* 179: 704–22
54. Buchanan, R. A., Kinkel, A. W., Goulet, J. R., Smith, T. C. 1972. *Neurology* 22: 126–30
55. Odar-Cederlöf, I., Lunde, P., Sjöqvist, F. 1970. *Lancet* 2:831–32
56. Reidenberg, M. M., Odar-Cederlöf, I., von Bahr, C., Borgå, O., Sjöqvist, F. 1971. *N. Engl. J. Med.* 285:264–67
57. Russell, M. A., Bousvaros, G. 1968. *J. Am. Med. Assoc.* 206:2118–19
58. Unger, A. H., Sklaroff, H. J. 1967. *J. Am. Med. Assoc.* 200:335–36
59. Gellerman, G. L., Martinez, C. 1967. *J. Am. Med. Assoc.* 200:337–38
60. Goldschlager, A. W., Karliner, J. S. 1967. *Am. Heart J.* 74:410–12
61. Voight, G. C. 1968. *Johns Hopkins Med. J.* 123:153–57
62. Scriabine, A. et al 1970. *Tox. Appl. Pharmacol.* 17:708–20
63. Wood, R. A. 1971. *Brit. Med. J.* 1:645
64. Cacace, L. 1973. *J. Am. Med. Assoc.* 244:1428
65. Blumsohn, D., Seabrook, M. 1970. *S. Afr. Med. J.* 44:1207–8
66. Helfant, R. H., Scherlag, B. J., Damato, A. N. 1967. *Circulation* 36:108–18
67. Fulop, M., Widrow, D. R., Colmers, R. A., Epstein, E. J. 1966. *J. Am. Med. Assoc.* 196:454–56
68. Grissom, J. H., Sy, B. G., Duffy, J. P., Dunea, G. 1967. *Brit. Med. J.* 4:34
69. Lieberson, A. D., Schumacher, R. R., Childress, R. H., Boyd, D. L., Williams, J. F. Jr. 1967. *Circulation* 36:692–99
70. Mixter, C. G. III, Moran, J. M., Austen, W. G. 1966. *Am. J. Cardiol.* 17:332–38
71. Puri, P. S. 1971. *Am. Heart J.* 82:62–68
72. Louis, S., Kutt, H., McDowell, F. 1967. *Am. Heart J.* 74:523–29
73. Merritt, H. H., Putnam, T. J. 1938. *J. Am. Med. Assoc.* 111:1068–73
74. Kutt, H., Winters, W., Kokenge, R., McDowell, F. 1964. *Arch. Neurol.* 11: 642–48
75. Levy, L. L., Fenichel, G. M. 1965. *Neurology* 15:716–22
76. Logan, W. J., Freeman, J. M. 1969. *Arch. Neurol.* 21:631–37
77. Glaser, G. H. 1972. *Antiepileptic Drugs,* ed. D. M. Woodbury, J. K. Penry, R. P. Schmidt, 219–26. New York:Raven. 536 pp.
78. Sparberg, M. 1963. *Ann. Intern. Med.* 59:914–30
79. Reynolds, E. H. 1972. *Antiepileptic Drugs,* ed. D. M. Woodbury, J. K. Penry, R. P. Schmidt, 247–62. New York:Raven. 536 pp.

Copyright 1974. All rights reserved

PREVENTIVE THERAPY OF TUBERCULOSIS

❖7099

Donald L. Brummer, M.D.
Medical College of Virginia, Health Science Division, Virginia Commonwealth University, Richmond, Virginia

INTRODUCTION

The thinking in the United States prior to the mid-1950s, as discussed by Ferebee (1), was that asymptomatic primary tuberculosis in children is a benign process, and indeed a healed primary tuberculous lesion was considered to grant a certain immunity against subsequent infection. Antimicrobial therapy was considered necessary only for a small group whose primary infection caused clinical illness. In an even smaller group the complications of infection such as miliary or meningeal tuberculosis would require heroic therapy. While on the children's chest service at Bellevue Hospital, Lincoln noted that no children receiving a regimen including isoniazid developed tuberculous meningitis as compared to children treated with regimens not containing isoniazid. It was apparently at her suggestion to the United States Public Health Service that the original controlled studies on a cooperative basis were undertaken (2, 3).

CONTROLLED STUDIES

Wallgren in Sweden had shown (4) that most complications of tuberculous infection occur within five months after diagnosis of the primary disease. The cooperative study in the United States was designed to utilize pediatric patients of thirty-two centers in the United States, Puerto Rico, Canada, and Mexico, and to determine in a large patient group in a controlled fashion whether or not a one-year course of isoniazid therapy could prevent the complications of the primary infection in the treated group compared with the placebo group. The thrust of the early studies was on what was called chemoprophylaxis or prevention, although current thinking regards this type of isoniazid use as treatment. The use of isoniazid is therefore referred to here as preventive therapy.

Early in the clinical trials it was decided to use isoniazid in household contacts, a group in which the known risk within a year of the diagnosis of the index case was high. Tuberculin nonreactors were also admitted to the study (5, 6) in an attempt to measure the effectiveness of isoniazid in preventing infection as well as disease.

The cooperative study of children in the thirty-two centers included 2750 cases collected from January 1955 through December 1957. The household contact trials included 2814 patients who were admitted to the study from October 1956 through April 1957 (5). A larger study of household contacts was undertaken after the pilot study. A total of 25,033 contacts to 5677 index cases were enrolled from January 1957 through December 1959 (6).

A large study in San Francisco reported by Curry (7) involved school age tuberculin reactors. For a year or more 4102 reactors were followed: 2910 in the isoniazid-treated group and 1192 in the control group. There is a slight built-in bias in Curry's study because the controls were those children whose parents did not give permission for preventive therapy. A number of other high risk groups were studied in the late 1950s and early 1960s, including inhabitants of several Alaskan villages where the tuberculosis was present in epidemic proportions (8, 9).

All the previous groups studied were presumed to represent at least relatively recently infected individuals. A study using elderly institutionalized mental patients who presumably had been infected many years before and who might not be expected to be exposed to new infection was undertaken (10). A very early trial of isoniazid among mental hospital patients with inactive lesions on chest roentgenograms was reported by Katz et al (18, 19). In the early 1960s a group of persons with lesions on chest roentgenograms presumed to represent healed tuberculous lesions was enrolled in a trial (6).

Except for the original group of children in the controlled trials, 30% of whom were lost to follow-up by 1967, the long term follow-up has been remarkably successful in these studies. The household contact study was more adequately prepared and the loss in that group was only 1.2% by 1967 after a period of eight to ten years. The studies in the mental patients revealed a loss to follow-up of only 0.6% six to ten years after admission to the trial. Only 2.2% of the inactive cases were lost after three to seven years.

Reports on studies in other countries include those among contacts in Japan (11), household contacts in Kenya (12), the Philippines (13), and contacts in the Royal Netherlands Navy (14). A study of Greenland villagers treated with a very different schedule of isoniazid administration is reported by Groth-Peterson et al (15) and Horwitz (16). A Tunisian study under the auspices of UNICEF, the WHO, and the Government of Tunisia is reported by Nybce et al (17).

RESULTS

Prevention of Infection

In those studies where skin tests utilizing 5 TU of PPD-S (purified protein derivative-standard) were performed at the beginning and end of the year of either therapy or placebo, there were minor and probably insignificant differences between the two groups. Of more importance was the clear demonstration in both groups of the booster effect of repeated tuberculin skin testing (20, 21).

Eradication of Infection

If one assumes that a negative tuberculin skin test reaction means that there are no living tubercle bacilli in the body and conversely that a positive skin test reaction means their presence, then reversion of a positive skin test to a negative should indicate that all living tubercle bacilli had been killed. The Public Health Service trials indicate that the majority of people who were skin test positive at the time of admission to the trials were still reactive at the end of the trial. There were slightly more reversions among the isoniazid-treated groups than among the placebo groups. The differences are consistent, but small, and are of questionable significance. A late skin test survey done on a small group of children ten years after the trial showed no significant difference in skin test reactions to 5 TU PPD-S between the isoniazid-treated group and the placebo group.

Houk et al (22) reported an epidemic aboard a United States Navy vessel where all tuberculin converters were placed on isoniazid for a period of 12 months. Of the 45 men who were known to have had negative tuberculin skin tests three months before exposure and subsequent conversion to positive, all had become skin test negative by the fifth month of therapy. The results of this study are unique. The study of a similar experience in the Royal Netherlands Navy (14) does not show comparable results (22).

Incidence of Tuberculous Disease

The study (2, 3) of preventive therapy in the asymptomatic primary infected children demonstrated 41 complications of infection over a ten-year follow-up period in the group given placebo; a rate of 30.2 per 1000. The children who received a year of isoniazid had 5 complications for a rate of 3.6 per 1000. There was no miliary disease or tuberculous meningitis in the group given isoniazid but there were ten children receiving placebo who developed one or the other. It was noted in the course of the trials that the expected complications did not occur in the year of therapy. Accordingly the question arose whether isoniazid administration merely delayed the onset of such complications by a year. Follow-up of the patients in the study strongly suggested that the protection from isoniazid lasted for a period of time longer than the year of therapy since the incidence of disease in the treated group in the year after therapy remained lower than in the placebo group.

The household contact studies in this country and elsewhere (5, 6, 11–14) demonstrated significant protection afforded by the year of isoniazid. The new case rate for the ten-year period was 15.4 per 1000 for the placebo group and 6.2 per 1000 for the isoniazid-treated group in the United States Public Health Service trials.

The Japanese experience (11) showed little difference in the case rate between the treated and the control group. The reasons for this are not clear but it has been suggested that rapid inactivation of isoniazid in the Japanese population may account for this. A small study among Japanese railroad workers showed a 68% reduction in the incidence of disease in the group on isoniazid (23).

The studies in the Philippines (13) and the Royal Netherlands Navy (14) corroborated the United States Public Health Service trial results.

The results reported by Curry's study of the San Francisco school children are even more impressive (7). He estimates that the rate of tuberculosis in the untreated group is sixty-one times as great as in the treated group. Even allowing for the bias in his study, this is a very substantial decrease in risk afforded by isoniazid.

The studies of patients with abnormal chest roentgenograms included patients with previously known active disease and those whose abnormalities were thought to represent pre-existing tuberculous disease. Significant protection was afforded in both groups by the administration of isoniazid. Differences between the Public Health Service study (10) and the New York State study (18, 19) are difficult to explain. However, both studies clearly demonstrate a decrease in risk afforded by isoniazid.

The Alaskan study (8, 9) demonstrated the same remarkable protection afforded by isoniazid. The study in Greenland (15, 16) showed that even intermittent therapy with isoniazid administered only twice weekly for two alternating three-month periods in the year of treatment afforded protection estimated to be in the neighborhood of 30%. The Tunisian study (17) presented too many problem areas and questions to allow easy interpretation. It seems likely that much of the recommended isoniazid was not taken.

The current status of the other modality for preventing tuberculosis, i.e. the use of BCG (Bacillus of Calmette and Guerin) vaccination to increase immunity to the disease, is summarized in the report of a conference (24) chaired by Barclay and held at the National Institutes of Health in October 1971. Barclay's summary ends by stating:

> If one is prepared to accept certain difficulties in the implementation of a BCG program, if one is willing to accept a certain cost in loss of the tuberculin skin test and if one is willing to focus the program in high risk groups, then significant beneficial protection can be achieved in a vaccinated population by using BCG strains currently in production for clinical use.

Millar (25) presents evidence suggesting that considering the low infection rates in the United States, the loss of the tuberculin skin test is too costly since it would seriously interfere with the tuberculosis control programs currently underway in the United States.

COSTS

Two studies have looked extensively at the costs of finding tuberculin reactors and treating them for a year with isoniazid in terms of time and effort as well as money. Katz & Kunofsky (26) conclude that preventive therapy in patients with inactive tuberculosis is justified in tuberculin reactors with reduced resistance such as those on adrenal corticoids, undergoing gastrectomy, with leukemia or Hodgkins disease, or diabetes mellitus, as well as in those who are household contacts and recent converters. They suggest that the effort expended among tuberculin-positive adolescents and in the child-centered programs is out of proportion to the results in terms of cases of tuberculosis prevented. Their paper is commented on editorially by Edwards (27) who disagrees with the statements concerning adolescents. She states that a school group is easy to test and that administration of isoniazid might be performed by the school authorities on a five day a week basis at relatively little cost in money or real effort and that the results of the year of isoniazid therapy justify whatever effort is necessary.

Moulding (28) calculated the number of persons in certain risk categories who must be treated to prevent one case of tuberculosis and then calculated the cost of preventing that one case of disease. His formulations indicate that 14.5 persons with inactive disease, 37.5 children with asymptomatic primary disease, 81.3 household contacts of active cases, 110.3 tuberculin-positive adolescents with normal chest roentgenograms, or 163.4 tuberculin-positive adults with normal chest roentogenograms must be treated to prevent one case. The cost in dollars to prevent one case in each of the above groups is respectively $824, $1612, $813, $4743, and $7026. Moulding suggests that on this basis he can recommend preventive therapy for all cases of primary tuberculosis in children and for all inactive cases of tuberculosis. He thinks the justification for giving isoniazid to all tuberculin-positive adults with normal chest roentgenograms is weak. He states in his summary that in the intermediate situations preventive therapy is probably justified.

ADVERSE REACTIONS AND TOXICITY

Whenever any medication is administered to a group of humans adverse reactions will be reported. Each of the trials utilizing isoniazid had its share of minor adverse reactions, which in some cases prompted discontinuation of the drug. As is usual in such studies a number of patients who were receiving placebo also reported similar reactions. These included gastrointestinal upset, skin rash, "sick feeling," and others not listed. The number, percentages, and rates of these reactions were low: less than 2% of the isoniazid group and about 1.5% of the placebo group (5, 6).

In the study on treatment of patients with inactive lesions (6), placebo reactions for which the placebo was discontinued occurred in 3.8% and for the group on isoniazid 6.6%. Neurological reactions including headache, dizziness, numbness, paresthesias, or mental symptoms were reported in 0.7% of the placebo group and 1.7% of the isoniazid-treated group. Jaundice was noted in the inactive lesion study group for the first time in the Public Health Service trials. Three white males over age 40 developed jaundice in the first two months of the trial. The previous studies involving children showed no evidence of hepatic toxicity.

Over the following years and as a result of the benefits shown in the Public Health Service trials, the United States Public Health Service and the American Thoracic Society officially recommended that isoniazid be administered to all people with positive tuberculin skin tests. Many thousands of individuals were placed on preventive therapy through their local health departments and by their private physicians. In the years 1965 to 1970 there were sporadic cases of hepatitis reported in adults receiving isoniazid. Scharer & Smith (29) reported that 10% of the patients receiving isoniazid showed elevations of serum glutamic-oxalacetic transaminase and serum glutamic-pyruvic transaminase but indicated that this change occurred early in the course of treatment and was, as a rule, a transient elevation that returned to normal even in the face of continued administration of isoniazid.

In the same year several open cases of pulmonary tuberculosis were reported among employees on Capitol Hill in Washington DC. A skin testing survey was performed and in February 1970 2321 Capitol Hill employees were started on preventive therapy with isoniazid. During the following nine months 19 of the 2321 manifested clinical signs of liver disease. Two of these died. The full story is reported by Garibaldi et al (30).

Following this outbreak of isoniazid-associated hepatitis, a committee was convened in March 1971. Their report (31) and an editorial (32) brought this to the attention of the medical community. The committee was presented material analyzing the Public Health Service trials by five-year groups. The patients in the trials for 1955 to 1960 were mostly children and showed no obvious hepatitis. The groups treated from 1960 to 1965 showed rare instances of hepatitis as noted before, with only three cases in the study of patients with inactive lesions and one case in an alcoholic silicotic patient in another study. The incidence of isoniazid-associated hepatitis was noted to be of significance in the years 1965–1970. Investigations were undertaken to determine if there was a foreign element in the isoniazid used in the later trials but no difference could be found. A recent suggestion is that the hepatitis may be due to a drug interaction between isoniazid and something as yet undetermined in the environment of those individuals who developed hepatitis (33). Most, but not all of the patients on isoniazid who developed hepatitis were known to drink excessive amounts of alcohol.

An example of drug interaction was reported by Murray (34) in 1962 when a group of epileptics were treated with isoniazid while receiving diphenylhydantoin (DPH) and phenobarbital. Because of competition for acetylization in the liver the usual doses of DPH gave blood levels too high for the patients who then became excessively drowsy and lethargic. No such specific interaction has been found to date to explain the association of isoniazid administration and hepatitis.

In view of the recent problem with isoniazid, the ad hoc committee on isoniazid and liver disease (31) recommended priorities for the administration of preventive therapy, placing adult males with positive tuberculin skin tests and negative chest roentgenograms last on the list of those individuals who should receive prophylaxis. A large group of patients in all categories and in a number of cities was placed in a special surveillance study of isoniazid and hepatitis. The results of this study are not yet available. The committee's recommendations are reiterated by Moss et al (35) who did intensive studies on five cases of isoniazid-associated hepatitis and recommended monthly clinical evaluation of patients receiving isoniazid.

SUMMARY

1. The studies all present overwhelming evidence that isoniazid therapy can prevent tuberculosis morbidity in a significant number of patients who take at least nine months of the medication.

2. Most of the United States Public Health Service controlled trials showed that the protection afforded by isoniazid therapy continued for at least ten years following the year of therapy.

3. Some studies (5, 6) suggest that infection as measured by conversion of a negative tuberculin skin test to positive can also be partially prevented in that fewer of the negative household contacts receiving isoniazid became positive than similar contacts receiving placebo.

4. Reversion of a positive tuberculin skin test to negative during the year of therapy occurred about equally in all the control studies.

5. Isoniazid resistance of the organism following preventive therapy has not been a problem (5, 6).

6. Minor adverse reactions are not significantly higher in patients receiving isoniazid than in the group receiving placebo.

7. Since 1965 isoniazid-associated hepatitis has been seen in adults in numbers and at a rate higher than in previous years. The reasons for this are not clear but the incidence is such that the toxicity may preclude groups of adults who are at low risk of developing tuberculosis from receiving whatever benefits isoniazid may afford. In groups at high risk of developing tuberculosis (31) enough benefit may occur from the use of isoniazid to outweigh the risk of hepatitis if proper surveillance is undertaken. It should be remembered that the risk of developing tuberculosis continues for a lifetime, while the risk of hepatitis is present only during the year of therapy.

Literature Cited

1. Ferebee, S. H. 1970. *Advances in Tuberculosis Research,* 17:28–106. Basel: Karger
2. Ferebee, S. H., Mount, F. W., Anastasiades, A. A. 1957. *Am. Rev. Tuberc.* 76:942–63
3. Mount, F. W., Ferebee, S. H. 1961. *N. Engl. J. Med.* 265:713–21
4. Wallgren, A. 1948. *Tubercle* 29:245–51
5. Mount, F. W., Ferebee, S. H. 1962. *Am. Rev. Resp. Dis.* 85:821–27
6. Ferebee, S. H., Mount, F. W. 1962. *Am. Rev. Resp. Dis.* 85:490–521
7. Curry, F. J. 1967. *N. Engl. J. Med.* 277: 562–67
8. Comstock, G. W. 1962. *Am. Rev. Resp. Dis.* 86:810–22
9. Comstock, G. W., Ferebee, S. H., Hammes, L. M. 1967. *Am. Rev. Resp. Dis.* 95:935–43
10. Ferebee, S. H., Mount, F. W., Murray, F. J., Livesay, V. T. 1963. *Am. Rev. Resp. Dis.* 88:161–75
11. Bush, O. B. Jr., Sugimoto, M., Fugii, Y., Brown, F. A. Jr. 1965. *Am. Rev. Resp. Dis.* 92:732–40
12. Egsmose, T., Ang'Awa, J. O. W., Poti, S. J. 1965. *Bull. W. H. O.* 33:419–33
13. Del Castillo, H. et al 1965. *Bull. Quezon. Inst.* 7:277–90
14. Veening, G. J. J. 1968. *Bull. Int. Un. Tuberc.* 41:169–71
15. Groth-Petersen, E., Gad, U., Ostergaard, F. 1960. *Am. Rev. Resp. Dis.* 81:643–52

16. Horwitz, O., Payne, P. G., Wilbek, E. 1966. *Bull. W. H. O.* 35:509–29

17. Nyboe, J., Farah, A. R., Christensen, O. W. 1963. *WHO/TB/Tech. Information/10*

18. Katz, J., Kunofsky, S., Damijonaitis, V., Lableur, A., Caron, T. 1962. *Am. Rev. Resp. Dis.* 86:8–15

19. Katz, J., Kunofsky, S., Damijonaitis, V., Lableur, A., Caron, T. 1965. *Am. Rev. Resp. Dis.* 91:345–50

20. Ferebee, S. H., Mount, F. W. 1963. Presented at Nat. Tuberc. Assoc. Ann. Meeting, Denver, Colorado

21. Narain, R., Nair, S. S., Rao, G. R., Chandrasekhar, P., Lal, P. 1966. *Bull. W. H. O.* 34:623–35

22. Houk, V. N., Kent, D. C., Sorensen, K., Baker, J. H. 1968. *Arch. Environ. Health* 16:46–50

23. Chiba, Y., Takahara, T., Kondo, K., Nagashima, A. 1963. *Bull. Int. Un. Tuberc.* 69:91–93

24. Immunization in Tuberculosis. 1971. *Fogarty Int. Center Proc. No. 14* DHEW Publication No. (NIH) 76068. 252 pp.

25. Millar, J. D. 1971. See Ref. 24, p. 41–48

26. Katz, J., Kunofsky, S. 1971. *Chest* 59: 600–5

27. Edwards, P. Q. 1971. *Chest* 59:589

28. Moulding, T. 1971. *Ann. Intern. Med.* 74:761–70

29. Scharer, L., Smith, J. P. 1969. *Ann. Intern. Med.* 71:1113–20

30. Garibaldi, R. A., Drusin, R. E., Ferebee, S. H., Gregg, M. B. 1972. *Am. Rev. Resp. Dis.* 106:357–65

3 . Brummer, D. L. et al 1971. *Report of the Ad Hoc Committee on Isoniazid and Liver Disease.* Atlanta, Ga.: Center Disease Control, U.S. Dep. Health Ed. Welfare

32. Brummer, D. L. 1971. *Ann. Intern. Med.* 75:643–44

33. Woolpert, S. F. 1972. *Am. Rev. Resp. Dis.* 106:281–82

34. Murray, F. J. 1962. *Am. Rev. Resp. Dis.* 86:729–32

35. Moss, J. D., Lewis J. E., Knauer, C. M. 1972. *Am. Rev. Resp. Dis.* 106:849–56

Copyright 1974. All rights reserved

MECHANISM OF ACTION OF HEMOGLOBIN

◆7100

Arthur Arnone, Ph.D.
Department of Biochemistry, College of Medicine, University of Iowa, Iowa City, Iowa

In the human red blood cell, the affinity of hemoglobin for oxygen shows a cooperative (sigmoidal) response to changes in oxygen pressure and is regulated by small changes in pH (the Bohr effect), interaction with D-2,3-diphosphoglycerate (DPG), and by combination with CO_2. This paper will briefly review some of the very recent X-ray and biochemical research relating these allosteric effects to hemoglobin structure. For more detailed and comprehensive treatments, references 1–5 should be consulted.

HEMOGLOBIN STRUCTURE

In horse, man, and many other mammals, the hemoglobin tetramer has a molecular weight of 64,500, consisting of two identical α chains of 141 amino acids and two identical β chains of 146 residues (6). The three-dimensional structure and packing of these subunits (termed α_1, α_2, β_1, and β_2) is now known in great detail for both oxy- and deoxyhemoglobin as a result of the classic crystallographic studies of Perutz and his colleagues (7–13). They showed that the α and β chains are remarkably similar in secondary and tertiary structure to myoglobin. This "myoglobin fold" is formed from eight helical segments in myoglobin and the β chain, and seven helices in the α chain. These helices (labeled A through H, with the short six-residue D helix missing in the α chain) fold to form a compact globular structure with hydrophobic residues constrained to interior positions and polar residues on the surface (9, 14, 15). All three chains form a highly specialized heme binding site from nonpolar portions of helices E, F, G, and H, as well as the CD corner. In particular, the heme iron atom is always coordinated with the unprotonated ring nitrogen of histidine F8, termed the proximal histidine. The nonpolar environment is credited with stabilizing the heme iron atom in the ferrous state and allowing it to combine reversibly with molecular oxygen (11, 14).

The quaternary structure of the hemoglobin tetramer (9, 11, 12) is characterized by a very stable region of contact at the $\alpha_1\beta_1$ (or the symmetry related $\alpha_2\beta_2$) interface, involving 34 residues in oxyhemoglobin and 32 residues in deoxyhemoglobin. There is very little movement along this interface in the transition between the oxy and deoxy structures. On the other hand, the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) interface has fewer interchain contacts (20 residues interact in deoxyhemoglobin, 19 in oxyhemoglobin), and shows a much larger shift on ligand binding with movements as large as 7 Å. There are no strong contacts between the two β chains in either the oxy or deoxy structure, and the $\alpha_1\alpha_2$ contacts are limited to four salt-bridges in deoxyhemoglobin (12, 13).

COOPERATIVITY OF OXYGEN BINDING

The theoretical models formulated to explain allosteric action have been based on the concept of conformational mobility. In the model of Monod, Wyman & Changeux (16), this flexibility manifests itself in a concerted transition between two conformational states differing in quaternary and tertiary structure, whereas the model derived by Koshland, Nemethy & Filmer (17) postulates a sequential transition involving intermediate conformations at partial ligand saturation. Evidence supporting some aspects of each of these models has been reported, but the mechanism of many allosteric proteins is probably a complex hybrid of both schemes (18, 19). In any event, the mechanisms of allosteric control ultimately must be related to the detailed atomic structures of individual allosteric proteins and their protein-substrate, protein-effector complexes.

In the case of hemoglobin, such a stereochemical mechanism has been proposed by Perutz to explain the cooperative nature of ligand binding, the Bohr effect, and organic phosphate effects (13). Perutz's theory, which is based on X-ray studies of normal and modified hemoglobins, may be summarized as follows. The quaternary structure of the hemoglobin tetramer is in equilibrium between the two stable subunit configurations observed in the X-ray studies, termed oxy and deoxy, or *R* and *T* following the nomenclature of Monod, Wyman & Changeux (16). In deoxyhemoglobin the equilibrium is greatly in favor of the *T*-state, which has low oxygen affinity, whereas fully oxygenated hemoglobin is much more stable in the *R*-state, which has a high affinity for oxygen. From a study of the atomic models of oxy- and deoxyhemoglobin, Perutz argues on stereochemical grounds that intermediate quaternary structures would be very unstable. These atomic models also show that in the absence of ligand the quaternary deoxy structure is stabilized (relative to the oxy structure) by six intersubunit salt-bridges involving the C-terminal residues of the α and β chains. Specifically, the C-terminal carboxyl group of each β chain interacts across the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) interface with lysine 40α, the C-terminal carboxyl group of each α chain interacts with the N-terminal amino group of the opposite α chain, and the guanidinium ion of the C-terminal arginine residue of each α chain forms a salt-bridge with the carboxyl group of aspartate 126 of the opposite α chain. In addition, the deoxy tertiary structure of each β chain includes an intersubunit salt-bridge involving the imidazole group of the C-terminal histidine (histidine 146β) and aspartate 94β.

In the deoxy state, the heme iron is about 0.75 Å out of the plane of the heme group because the high-spin ferrous iron atom has an atomic radius which is too large to fit in the center of the porphyrin ring. As oxygen reacts with deoxyhemoglobin, the heme iron of the reacting subunit becomes hexacoordinate and low spin, and its atomic radius is reduced by 0.17 Å allowing it to move into the plane of the porphyrin ring. Thus, a small change in atomic radius is amplified by the heme group to produce a three-quarter angstrom shift in the position of the iron atom. This movement triggers a series of small (and as yet, ill-defined) changes in the tertiary structure of the reacting subunits which include a decrease in the distance between the proximal histidine and the porphyrin ring, and a shift of the F helix towards the H helix. In both the α and β chains this shift results in the expulsion of the penultimate residue, a tyrosine, from a pocket formed by parts of helices F and H. This in turn forces a rather large movement in the adjacent C-terminal residue, rupturing (or possibly just weakening) the salt-bridges described above. The loss of these salt-bridges progressively destabilizes the deoxy structure, shifting the equilibrium between the R- and T-states in favor of the R-state in which all the salt-bridges are fully broken, and thereby increases the oxygen affinity of the remaining unreacted subunits. Perutz's mechanism is outlined in Figure 1.

Support for this stereochemical mechanism comes from studies of hemoglobin derivatives in which the C-terminal residues have been deleted. For example, the C-terminal residue of the β chain, histidine 146β, can be specifically removed by digestion of isolated β chains with carboxypeptidase B followed by recombination

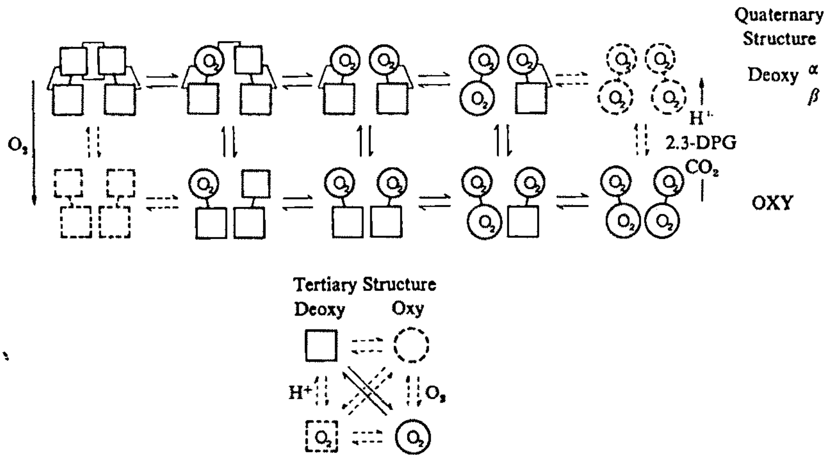


Figure 1 Diagrammatic sketch of Perutz's mechanism for cooperative oxygen binding. The subunit configuration in the top row of structures represents the deoxy quaternary structure at progressively increasing stages of oxygenation; the lower row represents the oxy quaternary structure. The fine lines between subunits represent the intersubunit salt-bridges involving the C-terminal residues, and the two heavy lines mark the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces. As oxygen reacts with hemoglobin the equilibrium between the two quaternary structures is shifted in favor of the oxy structure in which all the salt-bridges are broken and the remaining unreacted subunits have increased oxygen affinity. Very unstable forms are drawn with broken lines. From Perutz (13).

with α chains to produce des-(His 146β) hemoglobin (20). On the other hand, controlled digestion of native oxyhemoglobin with carboxypeptidase B removes the C-terminal residue of the α chain to yield des-(Arg 141α) hemoglobin (21). Des-(Arg 141α, His 146β) hemoglobin is prepared by carboxypeptidase B digestion of des-(His 146β) hemoglobin (22). Compared to native hemoglobin, des-(His 146β) hemoglobin shows reduced cooperativity and increased oxygen affinity, des-(Arg 141α) hemoglobin exhibits similar but more severe loss of function, and des-(Arg 141α, His 146β) is almost completely noncooperative (n, the Hill's constant, is 1.1) and has a very high oxygen affinity (22). Szabo & Karplus (23) have shown that to a first approximation these results are in agreement with the predictions of a mathematical model based on Perutz's mechanism.

THE ALKALINE BOHR EFFECT

The oxygen affinity of hemoglobin—as measured, for example, by the oxygen pressure required for half saturation—is pH dependent. Above pH 6.0 the oxygen affinity of hemoglobin increases with increasing pH, and as a result the transition from deoxy- to oxyhemoglobin is accompanied by the release of protons. That is, above pH 6.0 protons are preferentially bound to the deoxy (low affinity) structure. This can be rationalized if it is assumed that one or more titratable groups per $\alpha\beta$ dimer are changing pK values on oxygenation (24).

It is now firmly established that the C-terminal histidines of the β chains and the N-terminal amino groups of the α chains make a major contribution to the alkaline Bohr effect. If the reactive sulfhydryl group of cysteine 93β is blocked by reaction with N-ethylmaleimide to form N-ethylsuccinimide (NES) hemoglobin, 40% to 50% of the alkaline Bohr effect is lost (25, 26). X-ray studies showed that the salt-bridge between histidine 146β and aspartate 94β in deoxyhemoglobin is broken by the bulky NES group, implying that histidine 146β is an alkaline Bohr group. This was confirmed when des-(His 146β) hemoglobin was shown to have a halved alkaline Bohr effect (20). Just recently, Kilmartin et al have directly measured pK values for histidine 146β of 7.1 in carbon monoxyhemoglobin and 8.0 in deoxyhemoglobin by comparing the NMR spectra of normal and des-(His 146β) hemoglobin at different pH values (27). Thus, there would appear to be little doubt that histidine 146β is responsible for almost half of the alkaline Bohr effect.

Evidence for the role of the α chain N-terminal amino group in the alkaline Bohr effect comes from a hemoglobin derivative in which this α-amino group has been carbamylated by reaction with cyanate (28, 29). In both horse and human hemoglobin this derivative is characterized by a reduction of 25% in the alkaline Bohr effect. In contrast, there is no change in the alkaline Bohr effect when the N-terminal amino groups of the β chains are carbamylated (28, 29). By subtracting the Bohr effect curves of normal and α chain carbamylated hemoglobin, pK values of 7.3 and 7.7 have been estimated for the α chain N-terminal amino group in horse oxy- and deoxyhemoglobin (28). Similar calculations for human hemoglobin give approximate pK values of 7.1 in oxyhemoglobin and 7.6 in deoxyhemoglobin for this α-amino group (27, 29).

One of the most appealing aspects of Perutz's stereochemical mechanism is that it "naturally" accounts for the alkaline Bohr effect (13). Four of the eight salt-bridges observed to form only in the deoxy structure involved the terminal amino groups of the α chains and imidazole groups of histidines $146\beta_1$ and β_2. In oxyhemoglobin these groups are free to interact with the surrounding solvent and their pK values should be normal (i.e. close to 7.0), whereas in deoxyhemoglobin they are bound to carboxyl groups and should therefore have higher pK values. Consequently, as oxygen reacts with deoxyhemoglobin and these salt-bridges are broken, protons should be released. Conversely, as oxygen is transferred from oxyhemoglobin to the tissues, protons will be incorporated into these salt-bridges as they reform. Physiologically, this provides a major mechanism for transporting CO_2 from the tissues to the lungs by neutralizing the carbonic acid formed in the erythrocyte by the carbonic anhydrase catalyzed hydration of CO_2.

However, valine 1α and histidine 143β can account for only 75% of the alkaline Bohr effect at most. It has been calculated that if only one more alkaline Bohr group exists per $\alpha\beta$ dimer, it should have a pK of 6.4 in oxyhemoglobin and 7.4 in deoxyhemoglobin (27). Perutz has suggested histidine 122α as a candidate Bohr group since it is close to the guanidinium ion of arginine 30 of a neighboring β chain in oxyhemoglobin, but near the carboxyl group of aspartate 126 of the same α chain in deoxyhemoglobin (13). In agreement with the calculation mentioned above,this predicts that the pK of histidine 122α is lowered in oxyhemoglobin and raised in deoxyhemoglobin.

DPG BINDING

Glycolysis in the red blood cell is geared to the production of large amounts of D-2,3-diphosphoglycerate (DPG) (30). The reason for such a high DPG concentration (5 m*M*) was a mystery until the fundamental discovery by Benesch & Benesch (31) and Chanutin & Curnish (32) that DPG lowers the oxygen affinity of hemoglobin and thereby facilitates the unloading of oxygen to the tissues. Subsequent research showed that DPG binding was highly specific for deoxyhemoglobin with a stoichiometry of 1 mol DPG/mol hemoglobin tetramer (33). This one-to-one stoichiometry constrains the DPG binding site to the twofold symmetry axis of the hemoglobin tetramer (9, 34). The exact location of the DPG binding site was accurately predicted from biochemical studies (35, 36) and model building experiments (13), and then directly observed in an X-ray study (37) to be situated at the entrance to the central cavity between the N-terminal amino groups of the β chains.

This binding site is dominated by eight basic groups, four on each β chain (histidines 2 and 143, lysine 82, and the amino group of valine 1) related in pairs by the molecular twofold axis of symmetry. In the deoxyhemoglobin-DPG complex, the spatial arrangement of these cationic residues complements the anionic groups of DPG to form seven salt-bridges (see Figure 2). This is not the case, however, in oxyhemoglobin where the binding of oxygen changes the quaternary structure of the tetramer in a way that destroys this complementary stereochemistry (13). Thus a major effect of DPG binding appears to be a stabilization of the low affinity (deoxy)

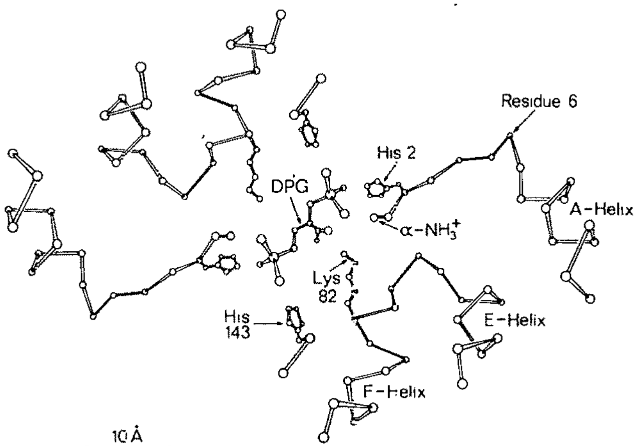


Figure 2 Sketch showing the binding of DPG to human deoxyhemoglobin. The stereochemistry of DPG complements the basic residues of the central cavity to form salt-bridges with valines 1 and histidines 2 and 143 of both β chains, and with lysine 82 of one β chain.

quaternary structure relative to the high affinity (oxy) quaternary structure. However, the X-ray studies have revealed that DPG binding also induces widespread distortions in the tertiary structure of the deoxy β subunits (37). These structural perturbations radiate from the DPG binding site and extend to the region of the β heme groups. Therefore DPG also may directly affect the intrinsic oxygen affinity of the β chains.

CO_2 INTERACTIONS

There appear to be two mechanisms for transporting CO_2 from the tissues to the lungs. In one case, hemoglobin serves to transport CO_2 indirectly by neutralizing the formation of carbonic acid via the alkaline Bohr effect. In the second scheme, CO_2 reacts directly with the α-amino groups of the hemoglobin tetramer to form carbamate ions, i.e.

$$RNH_2 + CO_2 \rightleftharpoons RNHCOO^- + H^+$$

Like the Bohr effect and DPG binding, the reaction of CO_2 with hemoglobin is "oxygen-linked." Thus more CO_2 is bound to deoxyhemoglobin than to oxyhemoglobin at a given CO_2 pressure, and conversely, the oxygen affinity of hemoglobin is decreased in the presence of CO_2 (38–40). In an elegant series of experiments, Kilmartin & Rossi-Bernardi studied the effect of CO_2 on the oxygen affinity of horse hemoglobin specifically modified at the α-amino groups by reaction with cyanate (28, 41). Their studies show that CO_2 has no effect on the oxygen affinity of hemoglobin when all four α-amino groups are carbamylated. However, only part of the oxygen-linked effect is destroyed when the α-amino groups of just the α or the β chains are blocked. This implies that the N-terminal amino groups are solely responsible for the oxygen-linked CO_2 binding, and that this effect is shared between the α and β chains. More recent experiments have confirmed these results for human hemoglobin (29).

From a structural point of view, these results imply that the formation of carbamate groups stabilizes the deoxyhemoglobin structure relative to the oxyhemoglobin structure. This prediction is easily rationalized in the case of the β chains, since the N-terminal amino groups of these subunits are part of a constellation of basic groups in deoxyhemoglobin designed to bind DPG (37). Hence, the formation of negatively charged carbamate groups should help to neutralize this region of high positive charge. In oxyhemoglobin these α-amino groups move to the vicinity of the C-terminus of the opposite β chain (9), and therefore the addition of a carbamate group may serve to destabilize the oxy structure.

The stereochemistry of CO_2 binding to the N-terminal amino groups of the α chains appears to be more complicated because these α-amino groups are interacting with the C-terminal carboxyl groups of the opposite α chains in deoxyhemoglobin (13). Since CO_2 reacts only with uncharged amino groups, these structural observations would seem to favor a higher CO_2 affinity for the oxy conformation (which contradicts the experimental findings mentioned above) unless the creation of negatively charged carbamate groups on the N-termini of the α chains induces a very stable state in deoxyhemoglobin or an unstable environment in oxyhemoglobin. Because of the highly exposed nature of these groups in oxyhemoglobin, the most likely possibility is the formation of a very stable carbamate salt-bridge with some basic group in deoxyhemoglobin. From model building studies, Kilmartin & Rossi-Bernardi suggest that the ε-amino groups of lysine 7α and lysine 139α could serve this purpose (28.)

PHYSIOLOGIC PROPERTIES OF HEMOGLOBIN

The physiologic properties of hemoglobin result from a complex integration of the individual allosteric effects described above. For example, since both DPG and CO_2 interact with the N-terminal amino groups of the β chains, they must compete for this binding site (42, 43). Solution studies have shown that more than 80% of the β chain carbamate groups are lost in the presence of DPG (44). On the other hand, DPG does not compete with CO_2 for the N-terminal amino groups of the α chains, and Brenna et al (44) have shown that both DPG and CO_2 are required to lower the oxygen affinity of pure hemoglobin solutions to that of whole blood.

The interaction of the anionic phosphate groups of DPG with the cationic residues of the β chains in deoxyhemoglobin must affect the pKs of both sets of groups in opposite directions. Experimental evidence shows that the alkaline Bohr effect is increased by as much as 50% in the presence of DPG (44, 45), which implies that the increase in the pKs of the four histidines and two α-amino groups at the DPG binding site dominates over the decrease in pK of the second ionization of the two phosphates. (In effect the binding of DPG to deoxyhemoglobin creates six alkaline Bohr groups and two acid Bohr groups.) In contrast, the combination of CO_2 with the α-amino groups of the α chains results in a large reduction of the alkaline Bohr effect (38, 44). The net Bohr effect is therefore a function of DPG concentration and CO_2 tension (44), with the former being more important at low pH (6.8) and the latter dominating at high pH (7.6).

Literature Cited

1. Braunitzer, G., Hilse, K., Rudloff, V., Hilschmann, N. 1964. *Advan. Protein Chem.* 19:1–71
2. Wyman, J. 1965. *Advan. Protein Chem.* 19:223–86
3. Antonini, E., Brunori, M. 1970. *Ann. Rev. Biochem.* 39:977–1042
4. Antonini, E., Brunori, M. 1971. *Hemoglobin and Myoglobin in their Reactions with Ligands.* London: North-Holland
5. Kilmartin, J. V., Rossi-Bernardi, L. 1973. *Physiol. Rev.* 53:836–90
6. Dayhoff, M. O., Eck, R. V. 1968. *Atlas of Protein Sequence and Structure.* Silver Spring, Maryland: Nat. Biomed. Res. Found.
7. Muirhead, H. Cox, J. M., Mazzarella, L., Perutz, M. F. 1967. *J. Mol. Biol.* 28: 117–56
8. Perutz, M. F. et al 1968. *Nature* 219: 29–32
9. Perutz, M. F., Muirhead, H., Cox, J. M., Goaman, L. C. G. 1968. *Nature* 219: 131–39

10 Perutz, M. F. 1969. *Proc. Roy. Soc. B.* 173:113–40

11. Muirhead, H., Greer, J. 1970. *Nature* 228:516–19
12. Bolton, W., Perutz, M. F. 1970. *Nature* 228:551–52
13. Perutz, M. F. 1970. *Nature* 228:726–39
14. Kendrew, J. C. 1962. *Brookhaven Symp. Biol.* 15:216–25
15. Perutz, M. F., Kendrew, J. C., Watson, H. C. 1965. *J. Mol. Biol.* 13:669–78
16. Monod, J., Wyman, J., Changeux, J. P. 1965. *J. Mol. Biol.* 12:88–118
17. Koshland, D. E. Jr., Nemethy, G., Filmer, D. 1966. *Biochemistry* 5:365–85
18. Hammes, G. G., Wu, C. W. 1971. *Science* 172:1205–11
19. Gutfreund, H. 1972. *Enzymes: Physical Principles.* London: Wiley
20. Kilmartin, J. V., Wcotton, J. F. 1970. *Nature* 228:766–67
21. Antonini, E., Wyman, J., Zito, R., Rossi-Fanelli, A., Caputo, A. 1961. *J. Biol. Chem.* 236:PC60–PC63
22. Kilmartin, J. V., Hewitt, J. A. 1972. *Cold Spring Harbor Symp. Quant. Biol.* 36: 311–14
23. Szabo, A., Karplus, M. 1972. *J. Mol. Biol.* 72:163–97
24. Wyman, J. 1948. *Advan. Protein Chem.* 4:407–531
25. Benesch, R., Benesch, R. E. 1961. *J. Biol. Chem.* 2236:405–10
26. Riggs, A. 1961. *J. Biol. Chem.* 236: 1948–54
27. Kilmartin, J. V., Breen, J. J., Roberts, G. C. K., Ho, C. 1973. *Proc. Nat. Acad. Sci. USA* 70:1246–49
28. Kilmartin, J. V., Rossi-Bernardi, L. 1971. *Biochem. J.* 124:31–45
29. Kilmartin, J. V., Fogg, J., Luzzana, M., Rossi-Bernardi, L. 1973. *J. Biol. Chem.* 248:7039–43
30. Rapoport, S. 1969. *Forsvarmedicin* 5: 168–74
31. Benesch, R., Benesch, R. E. 1967. *Biochem. Biophys. Res. Commun.* 26: 162–67
32. Chanutin, A., Curnish, R. R. 1967. *Arch. Biochem. Biophys.* 121:96–102
33. Benesch, R., Benesch, R. E. 1968. *Proc. Nat. Acad. Sci. USA* 59:526–32
34. Benesch, R., Benesch, R. E., Enoki, Y. 1968. *Biochemistry* 61:1102–6
35. Bunn, H. F., Briehl, R. W. 1970. *J. Clin. Invest.* 49:1088–95
36. Benesch, R. E., Benesch, R., Renthal, R. D., Maeda, N. 1972. *Biochemistry* 19: 3576–82
37. Arnone, A. 1972. *Nature* 237:146–49
38. Rossi-Bernardi, L., Roughton, E. J. W. 1967. *J. Physiol.* 189:1–29
39. Roughton, F. W. J. 1970. *Biochem. J.* 117:801–12
40. Bauer, C., Schroder, E. 1972. *J. Physiol.* 227:457–71
41. Kilmartin, J. V., Rossi-Bernardi, L. 1969. *Nature* 222:1243–46
42. Bauer, C. 1969. *Life Sci.* 8:1041–46
43. Pace, M., Rossi-Bernardi, L., Roughton, F. W. J. 1970. *Biochem. J.* 119: 67–68P
44. Brenna, O. et al 1972. *Hemoglobin and Red Cell Structure and Function,* ed. G. J. Brewer, 19–40. New York:Plenum. 556 pp.
45. Bailey, J. F., Bettlestone, J. G., Irvine, D. H. 1970. *J. Chem. Soc. A* 5:726

Copyright 1974. All rights reserved

CHARACTERIZATION OF AMYLOID

❖7101

George G. Glenner, M.D. and William D. Terry, M.D.
Section on Molecular Pathology, Laboratory of Experimental Pathology, National Institute of Arthritis, Metabolism, and Digestive Diseases and Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

INTRODUCTION

The nature of the unique proteinaceous deposits in tissues in both systemic and localized amyloidosis has eluded investigative efforts for well over a century. Recent chemical and immunochemical evidence has demonstrated that in many cases of amyloidosis, the amyloid fibrils, which constitute one of the distinguishing features of the deposits, have as their major constituent a protein of immunoglobulin origin (1–3). These findings substantiate the suggestions of several investigators (4, 5), most notably those of Osserman and co-workers (6–8). Amyloid fibrils of immunoglobulin origin are most frequently demonstrated in patients with multiple myeloma or other plasma cell dyscrasia and usually, but not exclusively, in patients with the "primary" (idiopathic) form of the disease. In these cases the major protein of the amyloid fibril has been shown to be a homogeneous light polypeptide chain (L) and/or its amino-terminal variable (V_L) fragment (3). Amino acid sequence analysis (1, 9, 10) and immunochemical investigation (2) have shown that these amyloid fibril proteins may be classified as either kappa- or lambda-type based on their chemical homology and/or sharing of their antigenic determinants with kappa or lambda Bence Jones proteins. In most cases further classification into subgroups, e.g. $V_{\kappa I}$, of these amyloid proteins has been possible (Table 1).

SOURCES OF AMYLOID FIBRIL PROTEINS

The source of the amyloid fibril protein has been the focus of several investigations. In one case of plasma cell dyscrasia without osteolytic lesions it has been shown by chemical analysis that the urinary Bence Jones protein and amyloid fibril protein have identical compositions and amino acid sequences and both, thus, have identical complete light polypeptide chains as their source (10). In a case of primary amyloidosis the use of an antiserum raised to the patient's lambda-type amyloid fibril protein detected a serum component that shared idiotypic antigenic determinants with the patient's amyloid fibril protein (11). With this idiotypic amyloid antibody as immunoabsorbant, it was possible to isolate from the patient's serum a protein (estimated concentration 0.1 mg/ml) that was shown by immunoelectrophoresis to have a restricted electrophoretic mobility and antigenic reactivity typical of a monoclonal lambda light polypeptide chain (3). Therefore, in a case of primary amyloidosis without overt cell dyscrasia in which the amyloid fibril protein was composed of a V_L fragment, the serum was found to contain an intact light chain that shared idiotypic determinants with the fibril protein. Analysis of the amyloid fibril protein from a case of isolated nodular pulmonary amyloidosis, a process in which dense plasmacytic aggregates surround the amyloid deposits and histiocytes infiltrate it, revealed the protein also to be a V_L fragment of a lambda light polypeptide chain (12). The source of the amyloid fibril protein, therefore, in cases of either occult or overt plasma cell dyscrasia, may be the circulating homogeneous light polypeptide chain of an immunoglobulin. In an individual case, however, the possibility of a homogeneous whole immunoglobulin protein or a variable portion of a heavy chain serving as a source of amyloid fibrils of immunoglobulin origin must still be considered. It is suggested that the source of the amyloid fibril in cases of localized tumor-like amyloid nodules may be a contiguous monoclonal plasmacytic infiltrate.

Table 1 Amyloid fibril proteins of immunoglobulin origin

Amyloid Protein	Major Protein(s)	Subgroup Classification		Clinical Classification
Cos (III)	V_L + L	$V_{\lambda I}$	(9)	Primary
Lua (L)	V_L	$V_{\lambda I}$	(9)	Isolated
Spe (VI)	V_L	$V_{\lambda II}$ or IV	(9)	Primary
Cra (XIV)	V_L	$V_{\lambda II}$ or IV	(9)	Primary
Art (VIII)	V_L + L	$V_{\kappa I}$	(1)	Secondary
Ale (X)	V_L	$V_{\kappa I}$	(1)	Primary
Tew	L	$V_{\kappa II}$	(10)	Plasma cell dyscrasia

MECHANISM OF AMYLOID FIBRIL FORMATION

Limited Proteolysis

Since in the majority of cases of amyloid fibril proteins of immunoglobulin origin the fibril was composed primarily of the amino-terminal fragment of a homogeneous light polypeptide chain, the possibility was considered that amyloid fibrils might be formed in these cases by proteolytic digestion of a monoclonal light chain. We applied a modification of a proteolytic technique (13) to cleave several lambda and kappa Bence Jones proteins into their variable (V_L) and constant (C_L) region halves (14). During the course of incubation of the lambda Bence Jones proteins with pepsin at pH 3.5 and 37°C, precipitates formed which stained with Congo red and when examined by polarization microscopy gave a green polarization color typical of amyloid deposits. Electron microscopic examination of the precipitates revealed them to be composed of fibrils indistinguishable from those of amyloid fibrils. X-ray crystallographic examination demonstrated the presence of a β-pleated sheet, a characteristic of amyloid fibrils (15), as the major protein conformation of these in vitro-created fibrils. The fibrillar precipitate from one of these preparations was studied in more detail and found to be derived from the amino-terminal variable portion of the parent Bence Jones protein. We concluded from these results that fibrils having the characteristics of amyloid fibrils could be created from some, but not all, Bence Jones proteins by proteolytic digestion. In other words, inherent in the structure of the V_L region of some light chains is the capacity to form β-pleated sheet fibrils when this region is cleaved from the intact light chain. Our findings, therefore, more closely associated amyloid fibrils with immunoglobulin proteins and suggested a proteolytic mechanism for fibril formation, presumably by the action of the lysosomal component of phagocytic cells acting on light polypeptide chains. In an independent study Tan & Epstein (16) reported that, in many instances, when light chain and Bence Jones proteins were incubated in the presence of renal lysosomal enzymes, polymers, insoluble under physiologic conditions, and composed of the whole light chain and the V_L and/or C_L halves of the chain, were formed. Subsequent studies revealed these to be fibrillar precipitates with the electron microscopic, tinctorial, and optical properties characteristic of amyloid fibrils (17). Our finding of both an intact homogeneous light polypeptide chain and its V_L fragment in several amyloid fibril preparations (Table 1) may, therefore, be explained by a mechanism in which a light chain copolymerizes with its amino-terminal fragment, created by lysosomal digestion, to form amyloid fibrils. The predominance of V_L fragments in these and other amyloid fibril preparations may be related to the preferential digestion of the C_L region at physiologic temperature (18).

If one mechanism for the production of amyloid fibrils is a limited proteolysis of certain light chains by phagocytic cells, the form in which a light chain exists in the serum may determine whether or not it will be excreted in the urine or digested to be deposited as amyloid fibrils. Our demonstration of large covalent polymeric forms of a circulating lambda light chain with idiotypic determinants in common with the amyloid fibril protein of the patient (3) and the descriptions of amyloidosis associated with circulating light chain polymers of high molecular weight (19) may have pathogenetic significance. The serum of a patient with such high molecular weight light chain polymers may become saturated at low solute concentration, particularly in sites where plasma concentration occurs, e.g. in capillaries. Under such circumstances these light chain polymers may precipitate and be processed by phagocytic cells, e.g. endothelial cells, in situ to form amyloid fibrils. It is of interest that lambda light chains are more frequently the source of amyloid fibril proteins and are more subject to polymer formation than kappa light chains.

β-Pleated Sheet Precursors

The demonstration by X-ray crystallography (15) and infrared spectroscopy (20) that the major protein conformation of amyloid fibrils is a β-structure has important implications in the pathogenesis of amyloid fibril formation and the nature of the precursor protein. The basis for the preponderance of light chain protein in the composition of amyloid fibrils of immunoglobulin origin is unknown. The most attractive hypothesis is that only certain light chain variable regions possess an "amyloidogenic" structure and that this structure is more prevalent in light than in heavy chains. The evidence favoring this hypothesis is the following. First, the ratio of kappa to lambda light chains in paraproteins (M-components) associated with amyloidosis (21) and in our series of amyloid fibril proteins (Table 1) is the inverse of the ratio in plasma cell dyscrasias without associated amyloidosis and in normal immunoglobulins. This suggests that lambda light chains have a structure more likely to become amyloid fibrils than do kappa chains. Second, kappa-type amyloid proteins share antigenic determinants with each other and with some, but not all, kappa Bence Jones proteins, while lambda-type amyloid proteins share antigenic determinants with each other and with some Bence Jones proteins, primarily of two variable region subclasses (2). These findings suggest that specific variable region sequences of immunoglobulin light chains may be required for the formation of β-pleated sheet fibrils during proteolytic cleavage.

Although we have emphasized intralysosomal proteolytic digestion of "amyloidogenic" light chains as a major mechanism of amyloid fibril formation from immunoglobulin proteins, in specific instances other mechanisms must be considered. Circumstances in which a light chain polymer may have selective affinity for an antigen can be envisioned (3). Under the conditions where a whole light chain forms an interchain disulfide dimer in solution, the size, chemical, and steric relationships between the two light chains would be similar to that present in a Fab fragment. Under these circumstances the interchain disulfide dimer could mimic a Fab fragment and have affinity for a specific antigenic determinant. In the process of binding an antigen a physical change in the light chain polymer may occur to produce a β-pleated sheet fibril (3).

FIBRIL PROTEINS OF NONIMMUNOGLOBULIN ORIGIN

The tinctorial and optical characteristics of amyloid deposits in tissues, e.g. Congo red polarization color, appear to depend upon the presence of β-pleated sheet fibrils (22). Some types of amyloidosis, therefore, could result from the tissue deposition of portions of immunoglobulins other than light chains, or of proteins other than immunoglobulins. Physicochemical mechanisms have been shown to induce the formation of β-pleated sheet fibrils from several naturally occurring proteins, e.g. insulin (23). It is, therefore, possible that the amyloid deposits associated with insulinomas (24) are of insulin derivation. A further major type of β-pleated sheet fibril protein, most frequently associated with cases of secondary amyloidosis, has been found to have a characteristic amino-terminal amino acid sequence not shared by any known immunoglobulin protein (25–28). Differences based on the presence or absence of arginine as the amino-terminal amino acid and differences in the molecular weights and/or the amino acid composition of the individual proteins reported indicate that, as with many amyloid fibril proteins of immunoglobulin origin, these proteins are derived from a larger precursor by proteolytic digestion. The nature of the protein of origin of this class of amyloid fibrils is, as of this writing, unknown. Two of the groups reporting this protein were also able to demonstrate, either by immunochemical methods (2) or peptide mapping (27), the presence of an immunoglobulin protein constituting a lesser proportion of their amyloid fibril preparations; this would imply that amyloid fibril formation in these cases also is linked to the deposition of the whole or a portion of an immunoglobulin molecule. It is, however, presently unknown whether the immunoglobulin protein component is homogeneous as in the case of amyloid fibrils of immunoglobulin origin.

THERAPEUTIC IMPLICATIONS

In those cases of amyloidosis in which the major protein of the fibrils is of immunoglobulin origin, therapy using immunosuppressive or cytotoxic agents to reduce the synthesis of the monoclonal protein has been suggested (29, 30). A therapeutic approach to those cases of amyloidosis in which the major protein of the fibrils appears not to be derived from an immunoglobulin may have to await knowledge of the cellular origin of this protein.

Literature Cited

1. Glenner, G. G., Terry, W., Harada, M., Isersky, C., Page, D. 1971. *Science* 171: 1150–51
2. Isersky, C., Ein, D., Page, D. L., Harada, M., Glenner, G. G. 1972. *J. Immunol.* 108:486–92
3. Glenner, G. G., Terry, W. D., Isersky, C. 1973. *Semin. Hematol.* 10:65–86
4. Magnus-Levy, A. 1952. *J. Mt. Sinai Hosp.* 19:8–9
5. Apitz, K. 1940. *Virchows Arch. (Pathol. Anat.)* 306:631–99
6. Osserman, E. F. 1959. *N. Engl. J. Med.* 261:1006–14
7. Osserman, E. F. 1961. *Am. J. Med.* 31: 671–75
8. Osserman, E. F., Takatsuki, K., Talal, N. 1964. *Semin. Hematol.* 1:3–86
9. Kimura, S., Guyer, R., Terry, W. D., Glenner, G. G. 1972. *J. Immunol.* 109: 891–92
10. Terry, W. et al 1973. *J. Clin. Invest.* 52:1276–81
11. Glenner, G. G. et al 1970. *Biochem. Biophys. Res. Commun.* 41:1287–89
12. Page, D. L., Isersky, C., Harada, M., Glenner, G. G. 1972. *Res. Exp. Med.* 159:75–86
13. Solomon, A., McLaughlin, C. L. 1969. *J. Biol. Chem.* 244:3393–3404
14. Glenner, G. G. et al 1971. *Science* 174: 712–14
15. Eanes, E. D., Glenner, G. G. 1968. *J. Histochem. Cytochem.* 16:673–77
16. Tan, M., Epstein, W. 1972. *Immunochemistry* 9:9–16
17. Epstein, W. V., Tan, M. 1972. *Arthritis Rheum.* 15:437
18. Seon, B.-K, Roholt, O. A., Pressman, D. 1972. *J. Biol. Chem.* 247:2151–55
19. Parr, D. M., Pruzanski, W., Scott, J. G., Mills, D. M. 1971. *Blood* 37: 473–84
20. Termine, J. D., Eanes, E. D., Ein, D., Glenner, G. G. 1972. *Biopolymers* 11: 1103–13
21. Pick, A. I., Osserman, E. F. 1968. *Amyloidosis,* ed. E. Mandema, L. Ruinen, J. H. Scholten, A. S. Cohen, 100–106. Amsterdam: Excerpta Medica. 462 pp.
22. Glenner, G. G., Eanes, E. D., Page, D. L. 1973. *J. Histochem. Cytochem.* 20: 821–26
23. Glenner, G. G., Eanes, E. D., Termine, J. D., Bladen, H. A., Linke, R. P. 1973. *J. Histochem. Cytochem.* 21:406
24. Porta, A. E., Yerry, R., Scott, R. F. 1962. *Am. J. Pathol.* 41:623–31
25. Benditt, E. P., Eriksen, N., Hermodson, M. A., Ericsson, L. H. 1971. *FEBS Lett.* 19:169–73
26. Ein, D., Kimura, S., Terry, W. D., Magnotta, J., Glenner, G. G. 1972. *J. Biol. Chem.* 247:5653–55
27. Levin, M., Franklin, E. D., Frangione, B., Pras, M. 1972. *J. Clin. Invest.* 51: 2773–76
28. Husby, G., Sletten, K., Michaelsen, T. E., Natvig, J. B. 1972. *Nature (New Biol.)* 238:187
29. Glenner, G. G., Ein, D., Terry, W. D. 1972. *Am. J. Med.* 52:141–47
30. Jones, N. F., Hilton, P. J., Tighe, J. R., Hobbs, J. R. 1972. *Lancet* 2: 616–19

Copyright 1974. All rights reserved

ISOLATED GROWTH HORMONE DEFICIENCY AND RELATED DISORDERS

❖7102

Thomas J. Merimee, M.D.
Department of Medicine, Boston University School of Medicine, Boston, Massachusetts

This brief discussion will consider those forms of dwarfism characterized either by a monotropic deficiency of human growth hormone (HGH) (a defect in synthesis and/or release), a defect in the ability of HGH to generate sulfation factor, or end organ subresponsiveness to exogenous or endogenous HGH. Although there is controversy over what term should be used to describe such dwarfs, the term ateliosis is historically correct, and a somewhat neutral term is useful if it is rigorously defined as in the preceding sentence.

Although interest in dwarfism has existed for centuries, it was not until 1902 that Guilford (1) proposed the first systematic classification based upon the simple observation that dwarfs appear to be of two major types: those with and those without obvious physical abnormalities. For the latter group, now known more popularly as midgets, Guilford used the term ateliosis (ateliosis = not arriving at perfection), since he considered this form of dwarfism a disorder in which growth and development were not arrested but in which both were retarded. He recognized, likewise, that some of these patients attained sexual maturity whereas others remained infantile, and for the former he coined the term "sexual ateliosis."

The most complete collection of familial cases of sexual ateliotic dwarfism prior to 1967 was compiled in 1912 by Rischbieth & Barrington (2). Rimoin was able to add to this collection when he recorded 64 individuals and 19 kindreds who could be classified as sexual ateliotics (3). In most of the kindred reviewed, autosomal recessive inheritance was suggested by the occurrence of affected siblings, both male and female, from unaffected parents. Using the Proband-Sib method for estimating the segregation ratio, data also indicated an autosomal recessive trait. The high frequency of consanguinity in the kindred studies was, likewise, consistent with this interpretation. Consanguinous matings occurred in 5 of 19 kindreds (26%) with sexual ateliosis (3).

Although our own data indicate that sexual ateliosis is usually inherited as a recessive trait, one family has been described in which the mode of inheritance is clearly dominant, and in two additional families neither simple recessive nor simple dominant inheritance could explain the pattern of inheritance (3, 4).

It is clear from the literature that isolated HGH deficiency can also occur, apparently, as a sporadic event (5, 6). Certainly most cases of panhypopituitary dwarfism are almost always nonfamilial. In one report Goodman, Grumbach & Kaplan summarized their findings in patients with HGH deficiency and no family history of dwarfism (7). It appeared to the authors that the distinction between single and multiple tropic hormone deficiencies was not constant in any of these patients. They noticed, for example, that thyroid function decreased in some patients with age. They were impressed with the high prevalence of breech deliveries and, accordingly, considered birth trauma as a possible factor leading to hypothalamic pituitary damage. This explanation might account for HGH deficiency occurring both sporadically and as a genetic disease.

It must be pointed out that in the familial variety considered here, there has been no tendency towards loss of other hormones. All of the sexual ateliotics we have studied were judged to have normal secretion of thyrotropin, gonadotropins, and ACTH. We can conclude from both a historic review and from our own data that sexual ateliosis occurs in an inherited and sporadic form. When inherited, it is usually transmitted as an autosomal recessive trait, with no tendency for a gradual loss of other pituitary hormones.

CLINICAL AND PHYSICAL FEATURES

We have examined a total of 35 patients (16 males and 19 females) with monotropic HGH deficiency whose ages range from 13 to 78. There are at least four separate subgroups which can be distinguished by laboratory data, but which cannot be clearly distinguished on clinical grounds (8). Thus, this clinical description covers all 35 individuals. Birth weights varied from 1.4 to 4.5 kg with a mean of 3.1 kg. Only two individuals weighed less than 2.7 kg at birth. Birth length ranged from 41 to 48.5 cm with a mean of 47 cm. In practically all instances, growth retardation was apparent by six months of age. Definite growth spurts of 7.5 to 20.5 cm occurred in the males at the time of puberty, but the females grew without an obvious spurt. Growth ceased at a somewhat earlier mean age of 21 yr in females versus a mean age of 25 yr in the males. Overall, there was considerable variation in the growth pattern. Two individuals, both male, grew until ages 36 and 34.

Although puberty occurs spontaneously in both sexes, its onset is usually quite delayed. Females noted menarche at ages varying from 12 to 27 yr; the mean age of menarche at 16 yr is ~4 yr later than normal. Puberty in males also occurs much later at a mean age of 19, again ~4–5 yr later than is normal.

On the basis of anthropometric data, our colleague, Dr. David Rimoin, assigned developmental ages to each subject relative to mean values obtained in normal children from birth to maturity (3). A comparison of these various developmental indices provides some objective information on the growth pattern of sexual ateliotics. The mean height age of the males is 8.7 yr with a range of 6.8 to 10 yr. The upper to lower segment ratio in the males ranges from 1 to 1.4 with a mean of 1.14. The mean normal ratio among adult American males is 0.91. The anthropometric ages, based on the upper to lower segment ratio, ranges in males from 2.3 to 9.5 yr, with a mean of 6.3 yr. Thus, in the males, the mean height is 2.4 yr greater than the mean anthropometric age. Since the upper to lower segment ratio is less than 1 by 10 yr, the affected males can be considered to have childlike proportions, i.e. they possess a relatively long trunk and short legs.

SPECIAL PHYSICAL CHARACTERISTICS

It has been emphasized previously that voice, skin, and facial features of dwarfs with a monotropic deficiency of HGH are quite often characteristic (3, 8). Such individuals often have a high pitched voice and childlike facies, with a weak, fully developed chin and a large prominent forehead. The skin is usually soft and finely wrinkled and appears to be thicker than normal. This wrinkling of the skin often begins early in life and the patients may, consequently, look prematurely old. In older subjects (greater than 40 yr), skin has greater thickness and elasticity than the skin of normal people of a similar age. Dermal and epidermal histology is found to be normal and the increased skin thickness is probably due to an increased amount of subcutaneous fat.

Data on physical characteristics can be summarized by stating that only one developmental index, the upper to lower segment ratio, is consistently abnormal. An important, and often neglected, point is that normal birth weight is characteristic; an abnormally low birth weight does not suggest pituitary insufficiency, but is more consistent with intrauterine growth retardation (3).

CLASSIFICATION OF GROWTH HORMONE DEFICIENCY AND RELATED DISORDERS

Evaluation of plasma growth hormone levels in our subjects was made in a variety of ways. However, all subjects were tested after at least two provocative tests: insulin-induced hypoglycemia and the infusion of the amino acid L-arginine (9, 10). A standard oral glucose tolerance test was also conducted in each individual. The use of these three procedures makes it possible to judge not merely the absence or presence of HGH, but also the ability to secrete insulin (i.e. in response to arginine and glucose). In addition, sensitivity to exogenous insulin can be judged after the insulin tolerance test.

At the time of this writing, the most satisfactory classification for states of isolated HGH deficiency and related disorders (sexual ateliotic dwarfism) appears to be the distinguishing of those dwarfs in whom immunoreactive HGH levels fail to increase after suitable provocative tests (true deficiency of HGH) from those dwarfs possessing HGH (8). A close look at the former population will reveal at least two subgroups.

Among individuals displaying HGH deficiency there is a group that shows the anticipated metabolic effects consequent upon lack of HGH, such as insulinopenia and insulin sensitivity—Group I (or classical monotropic HGH deficiency). However, there is a second subgroup also lacking HGH that does not display insulin sensitivity and, in addition, exhibits hyperinsulinism after glucose and arginine—Group II. Most of the members of Group I have a family history compatible with autosomal recessive inheritance, whereas the family history in Group II individuals indicates dominant inheritance. There are, in addition, dwarfs clinically indistinguishable from those with HGH deficiency and who display many of the metabolic features of HGH deficiency, i.e. insulinopenia and insulin sensitivity, but in whom HGH levels in plasma are normal or elevated. It is thought that the defect in this latter group resides in the inability to generate sulfation factor or perhaps some other metabolically reactive substance by which HGH exerts, in part or in whole, its biological action. These patients are referred to as Group III sexual ateliotics, or Laron dwarfs (11, 12). A fourth syndrome, Group IV sexual ateliosis, is represented by the African pygmies (13, 14) who have all of the expected metabolic features of HGH deficiency (insulin sensitivity and insulinopenia), but normal peripheral levels of HGH. Unlike the Laron dwarf, pygmies can generate sulfation factor. When the pygmy is treated with HGH, however, one sees none of the expected metabolic effects of HGH, such as nitrogen retention, increase of insulin secretion, or change in serum urea nitrogen. It seems reasonable that growth retardation in this group is probably consequent upon tissue insensitivity to either HGH or a product generated by HGH (15). To date, distinction between Group III and IV sexual ateliosis must be based on the levels of sulfation factor achieved after HGH administration and on plasma HGH response to glucose. Sulfation factor is low in Group III and normal in Group IV after administration of exogenous HGH (15). In addition, basal values of HGH are practically always elevated in Group III dwarfs and do not decrease when hyperglycemia is induced. The complete classification now proposed for these individuals is summarized in Table 1.

Since there is no satisfactory HGH releasing factor for use in humans, it is not possible to determine if the subjects in Group I or Group II can be further subdi-

Table 1 Classification of sexual ateliotic dwarfism

Group I:	Dwarfism associated with monotropic HGH deficiency; insulin hypersensitivity and insulinopenia are present.
Group II:	Dwarfism associated with monotropic HGH deficiency; normal insulin sensitivity and hyperinsulinism are present.
Group III:	Dwarfism associated with normal or high peripheral levels of immunoreactive HGH; metabolic profile consistent with HGH deficiency is present; sulfation factor after exogenous HGH is low.
Group IV:	Dwarfism associated with normal peripheral levels of immunoreactive HGH; metabolic profile consistent with HGH deficiency is present; sulfation factor after exogenous HGH is normal.

vided into those having a primary defect in the pituitary and those with a primary defect in the hypothalamus. This distinction will certainly be possible in the future.

In addition to the tentative classification of defects leading to the clinical picture of sexual ateliosis, there are several abnormalities related directly or indirectly to absolute or relative HGH deficiency that require emphasis.

Glucose Intolerance and HGH Deficiency

In sexual ateliotic dwarfs glucose tolerance is markedly impaired (16). This is true of most individuals over the age of 35 but only occasional individuals under the age of 20. An influence of HGH on insulin secretion is well established. Plasma insulin responses to glucose loading, for example, are exaggerated in acromegalics and markedly reduced in panhypopituitary dwarfs (16). The poor insulin responses in the Group I ateliotics could be restored by growth hormone administration, which further supports this concept (15). The cause of impaired glucose tolerance in Group II sexual ateliotics remains to be clarified. The major point is that gross glucose intolerance characterizes the majority of sexual ateliotics.

Hyperlipidemia and Growth Hormone Deficiency

Hypercholesterolemia and hypertriglyceridemia, like glucose intolerance, are common in sexual ateliotics; approximately 50% will show these abnormalities (17). Plasma lipoproteins have been analyzed in 12 dwarfs exhibiting a consistent elevation of plasma cholesterol greater than 280 mg%. This was associated, as one might predict, with a high level of plasma low density lipoproteins (LDL) and a prominent beta band on electrophoresis. This lipoprotein pattern seen in the sexual ateliotics most clearly resembles a Type IIb pattern (18). In this abnormality, LDL increases are the definitive finding, but very low density lipoproteins (VLDL) may also increase to a lesser degree. Since a substantial number of subjects lacking HGH have these abnormalities of their serum lipoprotein pattern, it seems likely that HGH modulates some aspect of lipoprotein metabolism. This may be a direct effect of HGH, or reflect faulty carbohydrate metabolism.

Fasting Homeostasis and HGH Deficiency

Sexual ateliotics do not tolerate well a prolonged fast. In the relative or absolute absence of HGH, blood glucose concentrations are not stabilized in a normal manner (19). With 72 hr of fasting, normal subjects stabilize glucose at a mean plasma concentration of approximately 65 mg% vs 39 mg% in dwarfs. Treatment with HGH prevents this abnormal decrease of plasma glucose concentration. Plasma free fatty acids (FFA), β-hydroxybutyrate, and acetoacetate will increase in dwarfs during a fast, since HGH is not required for either the mobilization of FFA or the subsequent conversion of FFA to ketone bodies.

Pregnancy and HGH Deficiency

Seven of the HGH-deficient females, by history, had normal pregnancies followed by normal lactation. Two sexual ateliotics were under our constant observation during their last trimester and postpartum period. Postpartum lactation occurred in both and each was capable of breast feeding their offspring. HGH was undetectable in both mothers throughout the period of lactation in both the basal state and following various stimuli for HGH secretion. HGH is, thus, not essential for postpartum lactation nor, by history, for normal placental function (20).

In summary, extreme short stature with normal but often delayed sexual maturation may occur either sporadically or as an inherited condition. In the absence of other causes (Turner's syndrome, Adrenogenital syndrome), at least four groups can be defined under the general term "sexual atelosis." An assessment of HGH secretion makes it possible to distinguish two groups lacking HGH and two possessing it. Distinction within these groups depends upon characterization of insulin secretion, insulin sensitivity, ability to produce sulfation factor, and responsiveness to exogenous HGH.

Literature Cited

1. Guilford, H. 1902. *Medico-Chirurgical Trans.* 85:305
2. Rischbieth, H., Barrington, A. 1912. *Treasury of Human Inheritance,* ed. J. Pearson, Pt. VIII, Sect. XV. London: Dulaw
3. Rimoin, D. L., Merimee, T. J., Rabinowitz, D., McKusick, V. A. 1968. *Recent. Progr. Horm. Res.* 24:365
4. Merimee, T. J., Hall, J. G., Rimoin, D. L., Fineberg, S. E., McKusick, V. A. 1969. *J. Clin. Invest.* 38:59a
5. Hewer, T. F. 1944. *J. Clin. Endocrinol.* 3:397
6. Wilber, J. F., Odell, W. D. 1965. *Metabolism* 14:590
7. Goodman, H. G., Grumbach, M. M., Kaplan, S. L. 1968. *N. Engl. J. Med.* 278:57
8. Merimee, T. J., Rimoin, D. L., Hall, J. D., McKusick, V. A. 1969. *Lancet* 1:963
9. Glick, S. M., Goldsmith, S. 1967. *Growth Hormone,* ed. A. Pecile, E. E. Muller, p. 84. Amsterdam: Excerpta Medica
10. Merimee, T. J. et al 1967. *N. Engl. J. Med.* 276:434
11. Laron, Z., Pertzelan, A., Mannheimer, S. 1966. *Isr. J. Med. Sci.* 2:152
12. Laron, Z., Pertzelan, A., Karp, M. 1958. *Isr. J. Med. Sci.* 1:883
13. Merimee, T. J., Rimoin, D. L., Rabinowitz, D., Cavalli-Sforza, L. L., McKusick, V. A. 1969. *Trans. Assoc. Am. Physicians* 81:221
14. Rimoin, D. L., Merimee, T. J., Rabinowitz, D., McKusick, V. A., Cavalli-Sforza, L. L. 1967. *Lancet* II:523
15. Merimee, T. J., Rimoin, D. L., Cavalli-Sforza, L. L. 1972. *J. Clin. Invest.* 51:395
16. Merimee, T. J., Rabinowitz, D., Rimoin, D. L., McKusick, V. A. 1968. *Metabolism* 16:1005
17. Merimee, T. J., Fineberg, S. E., McKusick, V. A., Hall, J. 1970. *J. Clin. Invest.* 49:1096
18. Merimee, T. J., Hollander, W., Fineberg, S. E. 1972. *Metabolism* 21:1053
19. Merimee, T. J., Felig, P., Marliss, E., Fineberg, S. E., Cahill, G. G. Jr. 1971. *J. Clin. Invest.* 50:574
20. Rimoin, D. L. et al 1968. *J. Clin. Endocrinol.* 28:1183

Copyright 1974. All rights reserved

ANTIARRHYTHMIC ACTIONS OF LIDOCAINE[1]

❖7103

Donald C. Harrison, M.D. and Ken A. Collinsworth, M.D.
Cardiology Division, Stanford University School of Medicine, Stanford, California

INTRODUCTION

Lidocaine was initially synthesized as an anesthetic agent in 1943, and its intravenous administration was attempted (1). Because of a poor activity spectrum and a high frequency of side effects, its intravenous use was abandoned and it became a widely accepted local anesthetic. In the 1950s, its properties as a cardiac antiarrhythmic agent were first recognized and it was used in the operating room by anesthesiologists for this purpose. In 1963, lidocaine's electrophysiologic and clinical effects were studied in postoperative cardiac surgery patients, with the conclusion that it was effective in abolishing ventricular arrhythmias when given intravenously, and that it produced less circulatory depression than procainamide when given intravenously to this group of patients (2).

With the development of coronary care units, the frequency of ventricular arrhythmias in the early postinfarction period was recognized. Lidocaine was then introduced for the treatment of ventricular tachycardia, and the concept documented that frequent ventricular premature beats often heralded more serious ventricular tachycardia and fibrillation (3–5). Thus, lidocaine was administered continuously to patients with premonitory dysrhythmias, with the idea of preventing more serious abnormalities. This concept has not been fully verified, but in a controlled study, Morgenson (5) showed a lower incidence of arrhythmias and death in a prophylactically treated group.

It was recognized that lidocaine had a short-effect half-life in man when given as a bolus (2). However, only after the development of a new gas chromatographic technique for measuring its concentration in blood could a rational program for administration be proposed (6). Harrison and associates (3, 7, 8) determined that the continuous administration of lidocaine in patients with infarction, at rates of 20–55 μg/kg/min, produced blood levels of 1.4–6.0 μg/ml between 2 and 8 hr after beginning the infusion (Figure 1). At these blood levels, the drug was effective in

---

[1] This work was supported in part by NIH Grants Nos. HL-5709, HL-5866, and 71-2482.

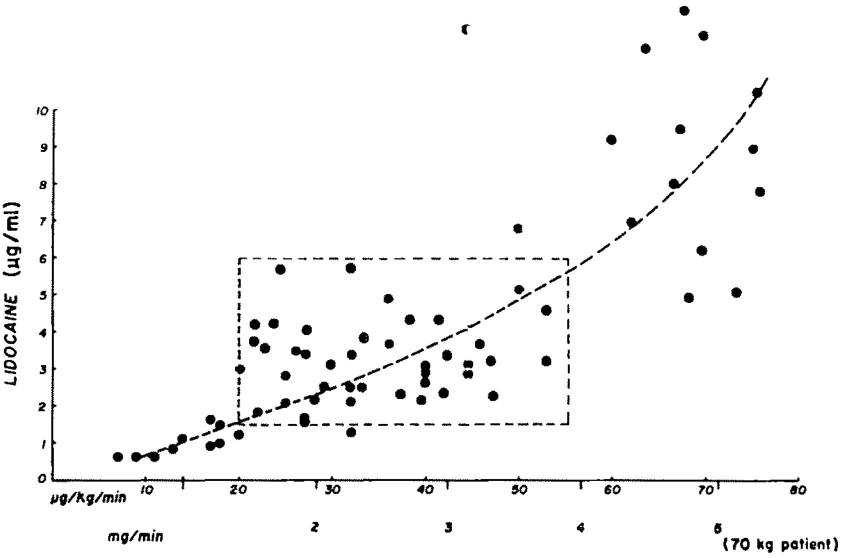


Figure 1 Relation between the rate of infusion of lidocaine and the blood level of lidocaine in patients receiving a constant infusion. All levels were measured after at least 2 hr of constant infusion in patients who were not in severe congestive heart failure or shock. The area enclosed within the rectangle is considered to be the effective therapeutic range. [Reprinted with permission of Harrison et al and *Modern Treatment* (7)]

abolishing or significantly reducing ventricular arrhythmias. Clear documentation that blood level is one of the primary determinants of effectiveness was therefore established. During the past eight years, lidocaine has become the most widely used antiarrhythmic drug in the coronary care unit, and recent studies have permitted a more comprehensive insight into its clinical pharmacology (9–12). The most relevant advances in understanding the action of lidocaine that have occurred in the past three years include: its electrophysiologic actions on cardiac tissues, its pharmacokinetics in man, its pathways for metabolism and excretion, and other routes of administration. These specific areas are reviewed in detail.

ELECTROPHYSIOLOGIC ACTION ON CARDIAC TISSUE

Several recent studies have defined the electrophysiologic properties of lidocaine (13–16), and although controversy remains, a number of points of general agreement exist. Lidocaine decreases automaticity of pacemaker tissue (15, 16), while leaving the diastolic threshold necessary for atrial and ventricular depolarization either unchanged or slightly elevated (15, 16). Fibrillation thresholds for ventricular muscle and Purkinje tissue are elevated in normal and ischemic hearts (17, 18). Action potential duration is probably shortened with high therapeutic concentrations, and the relative refractory period prolonged when compared to action potential duration (16). Lidocaine's effects on the maximum rate of depolarization and membrane responsiveness are the subject of controversy (15, 16). The differences in reported effects result from tissue-bath studies in which extracellular potassium concentrations varied. In low potassium concentrations, little effect on these parameters was noted (15), but in normal potassium solutions, membrane responsiveness and the rate of depolarization were depressed (16).

In the intact heart, A-V conduction time is not altered significantly by lidocaine in therapeutic concentrations (19). Lidocaine also reverses the electrophysiologic effects of toxic concentrations of digitalis (20). The basic electrophysiologic actions have been attributed to alteration of sodium-potassium pumping at the cell membrane (21, 22). It increases potassium conduction during spontaneous depolarization of Purkinje tissue, accounting for its ability to reduce automaticity (22).

Clearly, these electrophysiologic effects cannot be extrapolated directly to lidocaine's action in clinical practice, but they can be used to develop more precise studies in man and to determine a rationale for combinations of lidocaine with other available antiarrhythmic agents.

PHARMACOKINETIC STUDIES

Intravenous bolus injections of lidocaine provide peaks in blood concentrations rapidly, with therapeutic effects noted in 1–2 min (2, 3). There is an exponential decay with plasma half-times of 10–15 min. The height of the peak is determined by the effective volume of distribution, rate of administration, and the diffusion of lidocaine into tissue and extracellular compartments. Two-compartment models for distribution have been suggested (6, 9, 10). Thus, after a single injection, lidocaine's effect is of limited duration (2).

Prolonged infusions of lidocaine are generally administered after the first bolus has been given. In order to reach steady-state conditions 8–24 hr are required, depending upon metabolism and the effective volume of distribution (9, 10). Minimal effective therapeutic blood levels require 1–2 hr to achieve, unless a loading dose is administered (10). In normals, Boyes et al (9) found that 160 mg given as a bolus, followed by 4 mg/min by infusion, produced blood levels exceeding 1.4 μg/ml in a few minutes (9). If infusions were continued, steady-state levels of 2–4 μg/ml could be achieved in several hours.

Lidocaine is almost exclusively metabolized by microsomal enzymes in the liver (23–25), and in patients with reduced cardiac outputs, hepatic blood flow is generally decreased (11). Recent studies demonstrated a higher "steady-state blood level" for a given fixed infusion rate in patients with congestive heart failure (11, 12). Higher blood levels may have resulted from altered effective volumes of distribution, too, in these studies. After myocardial infarction, cardiac output and hepatic blood flow are likely reduced due to altered distribution of flow and effective volume of distribution reductions. Thus, utilizing Boyes' data and our studies, we have recently recommended a loading dose of 80 mg followed by an infusion rate of 2 mg/min as initial therapy after myocardial infarction when arrhythmias occur

(7). In patients with hepatocellular disease, altered metabolism has also been shown (12).

Several clinical implications are suggested by these pharmacokinetic studies. First, in order to achieve early therapeutic effects, a loading dose followed by an infusion is necessary. Second, when clearance is reduced by altered hepatic blood flow or reduced volumes of distribution, the doses must be reduced. Third, long periods are required to reach steady-state conditions and for any significant change in these conditions. Thus, altering the blood level during an infusion requires a new loading dose. Fourth, tapering lidocaine infusions are not necessary, since the blood level declines slowly as tissue compartments are mobilized.

METABOLISM AND EXCRETION

Lidocaine is almost exclusively metabolized in the liver (23, 24), although 5–10% may be excreted unchanged in the urine (25). The major pathway of metabolism is by oxidative N-deethylation to monoethylglycinexylidide, followed by hydrolysis to xylidine (24, 26). In man, conversion to 4-hydroxyxylidine occurs, with 70–85% of the metabolites being excreted in the urine (6, 25). Acid urine results in larger excretion. Other less important metabolites have also been reported. In animals, biliary excretion occurs, but in man this is apparently not an important pathway (26, 27).

OTHER ROUTES OF ADMINISTRATION

Oral administration of lidocaine has been attempted, but therapeutically effective blood levels have not been achieved (9, 28). After oral administration, a high incidence of mild central nervous system toxic effects has been observed, presumably due to metabolites, since gastrointestinal absorption results in passage through the liver and early metabolism.

Since lidocaine may be administered at home to patients experiencing myocardial infarction for protection during transport to the hospital, its blood level after intramuscular injection has been studied (28, 29). Injection of 4 or 6 mg/kg of 10% lidocaine produces effective blood levels in 5–10 min, persisting for 60–120 min (29). Injection into deltoid muscles produces higher blood levels more rapidly than injection into gluteal muscles and is the preferred site. Reduction in ventricular arrhythmias has been demonstrated after intramuscular injection (28) and correlated with effective blood levels.

OTHER ADVANCES

Lidocaine Resistance

Ventricular arrhythmias are occasionally resistant to the administration of lidocaine. Careful adherence to the pharmacokinetic principles outlined above reduces the incidence. However, when the arrhythmia is life-threatening and blood level measurements are available, larger and potentially toxic doses may be administered in an attempt to find a threshold for abolishing the arrhythmia. Procainamide administered in association with lidocaine appears to be the most appropriate combination drug program, based on electrophysiologic studies.

Lidocaine Toxicity

Serious side effects are focal and grand mal seizures, psychosis, and respiratory arrest. Less serious effects of drowsiness, paresthesias, disorientation, and agitation are commonly observed with large doses (30). Side effects cannot be directly related to blood levels, but good general correlation has been noted. True allergic reactions are uncommon (31).

Other drugs are frequently administered simultaneously with lidocaine, and drug interactions are common. Phenobarbital may enhance metabolism of lidocaine (32) and, by lowering hepatic blood flow, propranolol reduces metabolism of lidocaine (33).

Contraindications to Lidocaine

Idioventricular rhythms in complete heart block and extreme bradycardia contraindicate the use of lidocaine. The effect of lidocaine on A-V nodal conduction is controversial, with heart block reported (3) and accelerated A-V conduction (34). Further studies in man are needed.

CONCLUSIONS

Lidocaine is a safe and effective antiarrhythmic agent. Its action may be closely correlated with blood and tissue levels of the drug. Since its blood level is determined by the volume of distribution and its metabolism in the liver, disease processes that alter these should alter the kinetic programs for administration. In heart failure and shock, the dose must be reduced. Administration of a loading dose followed by continuous infusion produces therapeutically effective levels for treating and preventing ventricular arrhythmias in most patients with infarction.

Literature Cited

1. Lofgren, N. 1948. *Studies on Local Anesthetic Lidocaine, a New Synthetic Drug.* Stockholm: Ivan Halggstrom. (Reprinted by the Marin Press, Worcester, Mass.)
2. Harrison, D. C., Sprouse, J. C., Morrow, A. G. 1963. *Circulation* 28:486
3. Gianelly, R. E., von der Groeben, J. O., Spivack, A. P., Harrison, D. C. 1967. *N. Engl. J. Med.* 277:1215
4. Lown, B., Fakhro, A. M., Hood, W. B., Thorn, G. W. 1967. *J. Am. Med. Assoc.* 199:188
5. Morgenson, L. 1970. *Acta Med. Scand. Suppl.* 513:1
6. Keenaghan, J. B., Boyes, R. N. 1972. *J. Pharm. Pharmacol.* 150:454
7. Harrison, D. C., Alderman, E. L. 1972. *Mod. Treat.* 9:139
8. Harrison, D. C., Alderman, E. L. 1971. *Lidocaine in the Treatment of Ventricular Arrhythmias,* ed. D. B. Scott, D. G. Julian, p. 178. Edinburgh: Livingstone
9. Boyes, R. N., Scott, D. B., Jebson, P. J., Godman, M. J., Julian, D. G. 1971. *Clin. Pharmacol. Ther.* 12:105
10. Rowland, M., Thomson, P. D., Guichard, A., Melmon, K. L. 1971. *Ann. NY Acad. Sci.* 179:383
11. Stenson, R. E., Constantino, R. T., Harrison, D. C. 1971. *Circulation* 43: 205
12. Thomson, P. D., Rowland, M., Melmon, K. L. 1971. *Am. Heart J.* 82:417

13. Davis, L. O., Temte, J. U. 1969. *Circ. Res.* 24:639
14. Bigger, J. T., Mandel, W. J. 1970. *J. Clin. Invest.* 49:63
15. Mandel, W. J., Bigger, J. T. 1971. *J. Pharmacol. Exp. Ther.* 178:81
16. Singh, B. W., Vaughn Williams, E. M. 1971. *Circ. Res.* 29:286
17. Spear, J. F., Moore, E. N., Gerstenblith, G. 1972. *Circulation* 46:65
18. Wittig, J., Harrison, L. 1971. *Circulation* 44:II-85 (Abstr.)
19. Rosen, K., Lau, S., Weiss, M., Damato, A. 1970. *Am. J. Cardiol.* 25:1
20. Hilmi, K., Regan, T. 1968. *Am. Heart J.* 76:365
21. Arnsdorf, M. F., Bigger, J. T. 1972. *J. Clin. Invest.* 51:2252
22. Koerpel, B. J., Davis, L. D. 1972. *Circ. Res.* 30:681
23. Hollünger, G. 1960. *Acta Pharmacol. (Kobenhavn)* 17:356
24. Hollunger, G. 1960. *Acta. Pharmacol. (Kobenhavn)* 17:365
25. Eriksson, E., Granberg, P., Ortengren, B. 1966. *Acta Chir. Scand. Suppl.* 358:55
26. Breck, G. D., Trager, W. F. 1971. *Science* 173:544
27. Beckett, A. H., Boyes, R. N., Appleton, P. J. 1966. *J. Pharm. Pharmacol.* 18:76s
28. Fehmers, M., Dunning, A. 1972. *Am. J. Cardiol.* 29:514
29. Zener, J. C., Kerber, R. E., Spivack, A. P., Harrison, D. C. 1973. *Circulation* 47:984
30. Frieden, J. 1965. *Am. Heart J.* 70:713
31. Wellins, S. L. 1969. *Oral Surg.* 28:761
32. DiFazio, C. A., Brown, R. B. 1972. *Anesthesiology* 36:238
33. Branch, R. A., Shand, D. G., Wilkinson, G. R., Nies, A. S. 1973. *J. Pharmacol. Exp. Ther.* 184:515
34. Marriott, H. J. L., Bieza, C. F. 1972. *Chest* 61:682

Copyright 1974. All rights reserved

IMMUNOPATHOGENESIS OF SYSTEMIC LUPUS ERYTHEMATOSUS[1]

❖7104

David Koffler, M.D.
Department of Pathology, Mount Sinai School of Medicine and The Rockefeller University, New York, New York

In recent years, there has been considerable evidence accumulated concerning the pathogenesis of the renal and vascular lesions occurring in patients with systemic lupus erythematosus (SLE). The elucidation of mechanisms of tissue injury in SLE has contributed to a more rational basis for the treatment of the disease and has provided direct evidence that immunologic injury of the type induced experimentally in animals is operative in man as well. Although progress in the aforementioned areas has been impressive, the basic etiologic factors responsible for the initiation and perpetuation of the disease have eluded definition. As more information concerning precise immunological mechanisms is acquired, specific etiologies are suggested. In this review, we will cite recent advances concerning the immunopathogenesis of SLE and then discuss the hypothetical etiologies that derive from these observations.

Serologic studies indicate that a wide variety of antibodies directed against nuclear, cytoplasmic, and serum protein antigens are found in SLE sera (Table 1). Certain of these antibodies cross-react with bacterial and viral polynucleotides, raising the possibility that exogenous as well as native or altered tissue antigens may serve as immunogens. The antibodies directed against native DNA (NDNA) were among the first to be described in the sera of patients with SLE (1–3) and have been particularly useful diagnostically and as an indicator of disease activity. Several different techniques have been utilized for the detection of anti-NDNA antibodies, including agar gel diffusion (3, 4) and more sensitive techniques allowing the quantitation of anti-NDNA antibodies, which include complement fixation (1), hemagglutination (5–7), and tests using labeled DNA in Farr-type assays (8–10), and solid phase radioimmunoassays (11, 12). The latter tests are highly sensitive, and the detection of low titers of anti-native DNA antibodies must be interpreted with caution in view of the non-antibody type interaction of the DNA molecule with

---

[1] Supported by US Public Health Service Grants AM 13721 and AM 04761.

basic substances. Assays that are not solely dependent on the primary interaction of DNA and gamma globulin indicate that anti-NDNA antibodies are predominantly found in patients with SLE (5, 6), although patients with certain forms of chronic hepatitis and occasionally rheumatoid arthritis may manifest anti-NDNA antibodies (13). In contrast, antibodies reactive with single-stranded DNA (SDNA), which are present in high incidence in patients with SLE, are also found with considerable frequency in other disease states (5, 6). Both anti-NDNA and anti-SDNA antibodies appear to be heterogeneous directed against multiple determinants of NDNA and SDNA (4, 6, 14–19). Most anti-NDNA antibodies react as well or more avidly with SDNA than with NDNA, and only rare antibodies have unique specificity for NDNA (6). It has been suggested that SDNA may be a major immunogen for both types of antibodies. A third group of antibodies involving DNA are directed against nucleoprotein (20–22) and require both DNA and protein moieties for their interaction. These antibodies have not been studied as extensively because of difficulties in developing simple assay systems allowing quantitation of anti-nucleoprotein antibodies. Particulate and soluble nucleoprotein may be used for detecting antibodies by agar diffusion and complement fixation. Recently, a labeled nucleoprotein radioimmunoassay has been utilized for the quantitation of both anti-nucleoprotein and anti-NDNA antibodies (23). The majority of sera tested contained both types of antibody simultaneously, and the rises in the titer of anti-nucleoprotein antibody appeared to parallel that of anti-NDNA antibody. Several antibodies directed against soluble antigens present in nuclear extracts obtained by incubation of nuclei with saline have been characterized. Two antigens, Sm (24) and ribonucleoprotein (25–27), have been identified and appear to exist in the nuclear extract as a molecular complex with independent specificities (28).

In addition to the antibodies reactive with ribonucleoprotein in nuclear extracts, multiple antibodies reactive with single-stranded polyribonucleotides, double-stranded RNA, and cytoplasmic ribosomes have been demonstrated. Anti-ribosomal antibodies have been observed in patients with severe renal disease (29), whereas other anti-cytoplasmic antibodies have been found in lower incidence and are not well characterized (30, 31). Anti-RNA antibodies have also been demonstrated using labeled Poly I•Poly C (32–34) as well as hemagglutination tests (5, 6). These antibodies are also a heterogeneous group, some of which appear to be directed against double-stranded RNA (DSRNA). Although reactivity with reovirus RNA has been found (33), it is not clear if this represents a unique specificity of these antibodies or a cross-reaction involving double-stranded RNA from tissues or determinants of single-stranded RNA. Certain of these antibodies have been shown to cross-react with single-stranded Poly A, as well as in some cases with determinants on single-stranded DNA (6). Recent studies indicate that lymphocytotoxic antibody activity is also present in the sera of patients with SLE (35–39). Data from these studies suggest that these antibodies have T-cell (39–42) and possibly HLA specificity (35, 36). The significance of these antibodies in the pathogenesis of the disease remains to be elucidated, although it has been suggested that such antibodies may play a role in the impairment of delayed hypersensitivity, which has been observed in patients with SLE (43–47). In addition, a "shedding phenomenon" (39,

Table 1 Antibodies in SLE sera

I. Antibodies to nuclear constituents
    a. Native DNA
    b. Single-stranded DNA
    c. Nucleoprotein
    d. "Carbohydrate-Protein" antigens, Sm antigen, and others
    e. RNA-protein (ENA)

II. Antibodies to cytoplasmic constituents
    a. Ribosomes (RNAase sens.)
    b. Carbohydrate antigens
    c. Lipid antigens

III. Antibodies to gamma globulin

IV. Antibodies to clotting factors

V. Red cell antibodies

VI. Platelet antibodies

VII. Lymphocyte antibodies
    a. T-cell determinants
    b. ? Histocompatibility antigens

VIII. RNA antibodies
    a. Single-stranded polyribonucleotides (Poly A)
    b. Double-stranded polyribonucleotides

40) observed in vitro involving lymphocyte surface antigen and antibody may contribute to immune complex formation in vivo, although further studies are required to substantiate this hypothesis.

Serial studies with selected antibodies and circulating antigens have implicated several polynucleotides and gamma globulin in the formation of humoral immune complexes. NDNA (6, 14, 48) and SDNA (6, 49, 50) are major systems involved and in a lesser number of cases, ribonucleoprotein appears to be implicated. Serial studies of anti-nucleoprotein antibodies indicate that increases in titers of those antibodies are also closely related to clinical activity (23). In addition, cryoglobulins related to gamma-globulin–anti-gamma-globulin complexes have been found to occur in serum, as well as complexes precipitable with $C1_q$ globulin (51–55).

Increased titers of antibodies to NDNA are most closely correlated with clinical activity and the formation of complexes as suggested by the close relationship with depression of serum complement levels (14, 48) (Figure 1). SDNA antibodies also occur intermittently during periods of disease exacerbation, although these antibodies are less well correlated with periods of clinical activity than are anti-NDNA antibodies (Figure 2). Serial studies of a group of 25 patients followed for periods of six months to four years indicated that in most patients peaks of anti-NDNA and anti-SDNA antibody activity occurred at some time during the course of the disease (6) (Table 2). Both native and single-stranded DNA antigen have been found to alternate with the presence of circulating antibody in SLE patients. NDNA and SDNA have been demonstrated in serum by agar gel diffusion techniques (14, 50), counterimmunoelectrophoresis (56), as well as hemagglutination inhibition (50) with antibodies obtained from human sera and rabbit antisera. The hemagglutination inhibition system has allowed the quantitation of SDNA, and levels as high as 100–250 mg/ml have been found in selected sera obtained from serial studies (Figure 3). Several observations support the hypothesis that antigen-antibody complexes are formed in serum during the course of the disease. The alternating appearance of antigen and antibody for both the NDNA and SDNA systems, the

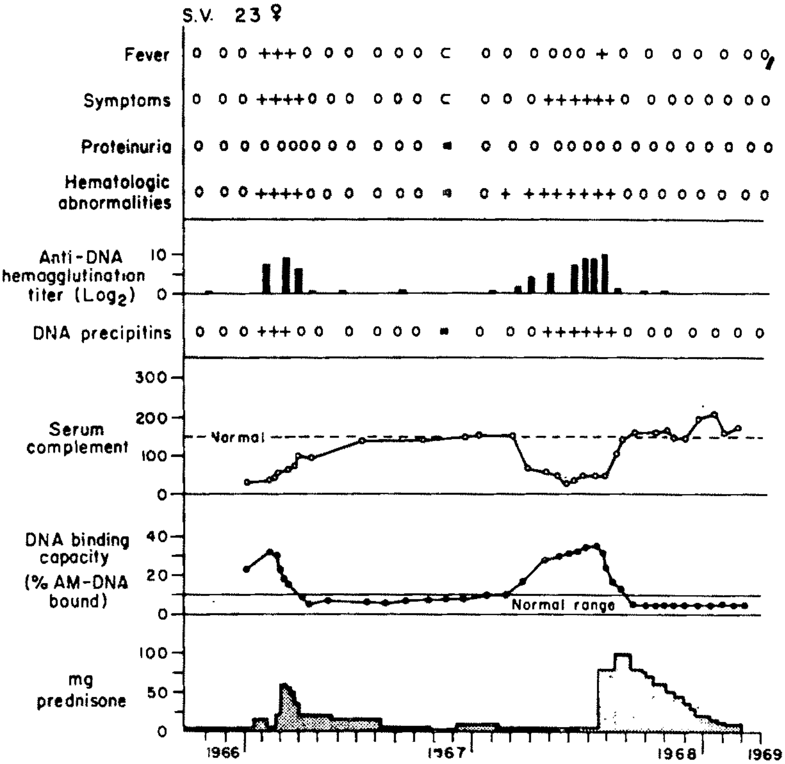


Figure 1 Serial study of patient S.V. showing two periods of clinical exacerbations associated with increases in titer of anti-NDNA antibodies and serum complement depression. Antibodies were assayed by agar gel precipitation, hemagglutination, and ammonium sulfate precipitation test using labeled DNA (49).

simultaneous demonstration of antigen and antibody in sera (50), and the recent studies of SLE sera which indicate that deoxyribonuclease treatment results in an increase in the titer of anti-NDNA antibodies (57) support this hypothesis. In certain patients a prolonged period of persistence of SDNA, extending four to six months, was noted. Therefore, although antibody activity appeared to correlate with clinical exacerbation in many cases, it was apparent that antigen excess could obscure the presence of antibody and probably contribute to the formation of tissue-localizing immune complexes in the absence of overt clinical activity. In addition to the aforementioned anti-NDNA–NDNA and anti-SDNA–SDNA complexes, agar gel diffusion studies indicated that anti-NDNA–SDNA complexes may also be present in the serum of an SLE patient (50). One serum in which free SDNA was found also contained precipitating anti-NDNA antibodies, suggesting that the native sites on the SDNA molecule were completely saturated and therefore a state of anti-NDNA antibody excess existed in the presence of excess SDNA.

The source of NDNA and SDNA in serum remains a subject for speculation. Inasmuch as both these antigens are found in serum, it is likely that they are

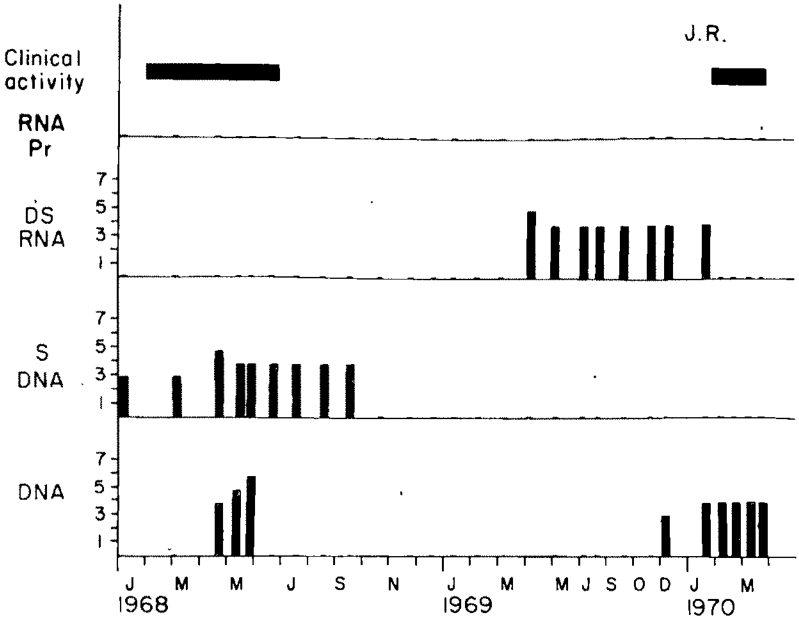


Figure 2 Two episodes of clinical activity associated with rises in anti-NDNA antibody titers. Steroid treatment was associated with a rapid decline in clinical symptoms with a concomitant decrease in anti-NDNA antibody. Anti-SDNA antibodies persisted after clinical symptoms abated. Anti-DSRNA antibodies appeared during a clinically quiescent period. Note the absence of anti-ribonucleoprotein antibodies in all sera tested (6).

Table 2 Serial studies of 25 patients with SLE[a]

	DNA	SDNA	Antibody Poly A · Poly U	RNA Pr
All sera positive	3	3	2	7
All sera negative	3	2	6	8
Peaks of antibody activity	19	20	17	10

[a] From reference 6.

potentially immunogenic in SLE patients, who may have an increased propensity for antibody formation to polynucleotide antigens. NDNA has been found at high levels in the sera of disease states in which no antibody is found (58). SDNA, in contrast, occurs at highest levels in those patients with high incidence of anti-SDNA antibody (50) (Table 3). Although a bacterial or viral source for NDNA or SDNA cannot be excluded, the large concentrations of polynucleotide found in serum suggest that the primary source is endogenous. The influence of steroid therapy on the release of NDNA antigen into the circulation has been cited (59), although a similar correlation between steroid treatment and the appearance of SDNA antigen has not been observed.

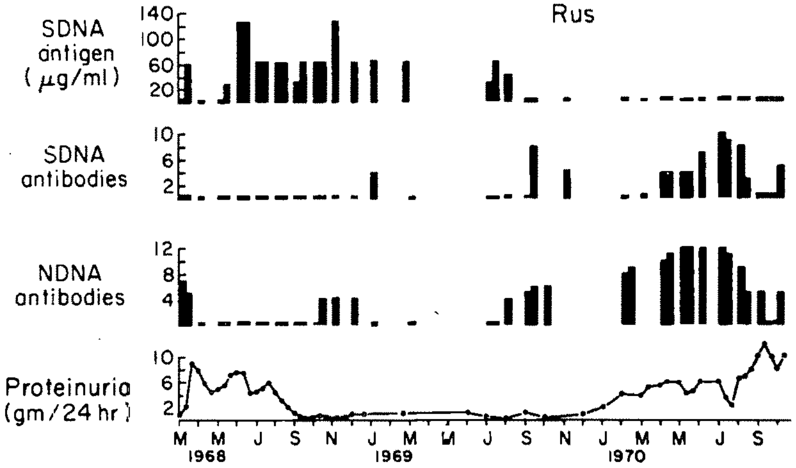


Figure 3 Rus. Patient with SLE followed for a period of 2 1/2 years, during which two major episodes of glomerulonephritis occurred. The first exacerbation of renal disease was associated with a brief rise in titer of anti-NDNA antibodies and no demonstrable anti-SDNA antibodies. The second episode of renal disease was associated with high titers of both anti-SDNA and anti-NDNA antibodies. SDNA was detectable in sera during the first episode of renal disease, whereas none was observed during the second episode. (50).

Table 3 SDNA antigen in human sera[a, b]

		Incidence of		
	Number of Sera	SDNA Antibody	SDNA Antigen	SDNA
		% positive	% positive[c]	μg/ml
SLE	60	52	55	23.0[d]
Rheumatoid arthritis	54	24	49	18.6
Chronic glomerulonephritis	40	5	18	5.9
Leukemia	19	5	39	9.6
Malignant tumors	20	0	15	7.5
Hospital diseases	99	5	20	6.0
Normal human sera	56	4	4	5.6

[a] From reference 50.
[b] Hemagglutination inhibition assay.
[c] Incidence in sera without detectable antibody.
[d] Mean concentration.

Anti-ribonucleoprotein antibodies exhibit a different pattern in the serial studies from those of anti-NDNA and anti-SDNA antibodies (6) (Table 2). Rather than occurring in peaks of activity, the majority of patients studied either exhibited elevated titers of this antibody in most sera or showed a complete absence throughout their course, although some patients did show peaks of activity. Recent studies indicate that ribonucleoprotein antigen is present in SLE sera (60) and may alternate with the appearance of ribonucleoprotein antibody. Evidence from tissue studies to be discussed subsequently further implicate this system in the formation of tissue-localizing antigen-antibody complexes. Antibodies reactive with double-stranded RNA (DSRNA) have also been shown to occur in peaks of activity (6), which occasionally arise concomitantly with anti-SDNA and anti-NDNA antibodies. Peaks of these antibodies also occur during clinically quiescent periods, and no direct relationship to disease activity has been observed. Preliminary evidence indicates that DSRNA antigen may be found during serial studies of SLE patients' sera (61) and may alternate with anti-DSRNA antibody. The serological status of these immune complex systems is currently being investigated.

Serum cryoglobulins have been demonstrated during periods of acute disease activity, especially in patients with evidence of nephritis (51, 52). The phenomenon of cryoglobulinemia appears to represent an in vitro precipitation of complexes closely related to the gamma-globulin–anti-gamma-globulin system. Studies with the $C1_q$ globulin component of complement, which forms precipitin bands with soluble immune complexes and aggregated gamma globulin in agar gel (62), suggest that a relation exists between cryoprecipitates and $C1_q$ precipitin reactions in SLE sera (53–55). Isolation of precipitates from SLE sera utilizing $C1_q$ indicates that high molecular weight complexes containing IgM anti-gamma globulins are present (55). Although the $C1_q$ reactants and cryoglobulins usually appear concurrently with rises in titer of anti-DNA antibodies, in several patients anti-DNA antibodies were absent in the presence of serum cryoglobulins, complement depression, and proteinuria (Figure 4). In addition to high molecular weight complexes, $C1_q$ globulin is capable of demonstrating low molecular weight complexes which are associated with hypocomplementemia and clinical activity but do not appear to be correlated with the presence of nephritis. The low molecular weight complexes are resistant to reduction and alkylation and are unreactive with anti-gamma globulin. The complexes have not been characterized and may represent polyanions such as polynucleotides, heparin, or lipopolysaccharides, which are reactive with $C1_q$ globulin. Approximately 75% of hypocomplementemic SLE sera contain $C1_q$ reactants that may be characterized as high or low molecular weight. The former are related to the gamma-globulin–anti-gamma-globulin system, whereas the role of the latter is unknown (55).

Serological studies, therefore, have suggested that different types of immune complexes are formed in the sera of patients with SLE. Tissue studies performed

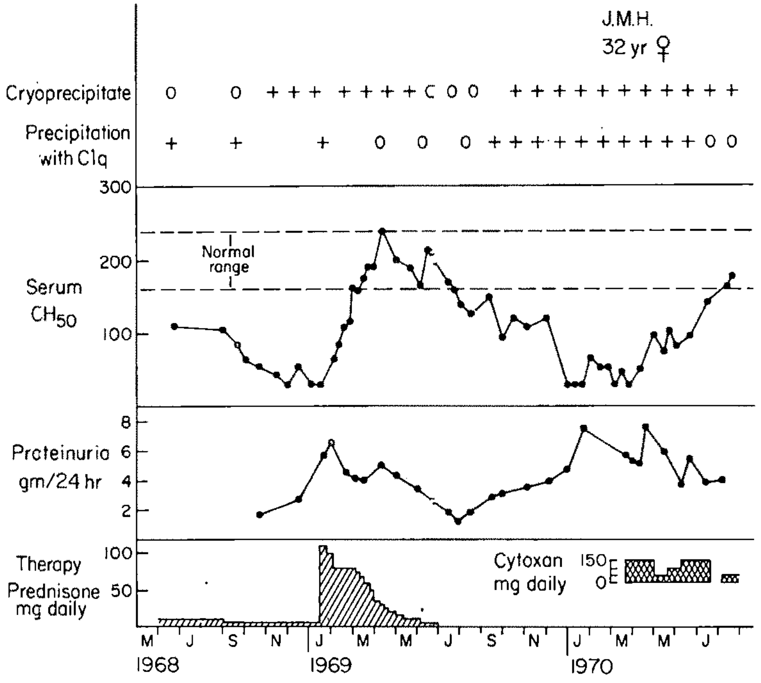


Figure 4 Serial study of an exceptional SLE patient (J.M.H.) showing two broad periods of clinical activity with renal disease which were associated with the presence of cryoprecipitins and $C1_q$ reactivity in the serum. No antibodies to polynucleotides were present (55).

on renal biopsies and kidneys obtained at autopsy have provided further evidence concerning the heterogeneous nature of antigen-antibody complex deposits and helped to confirm the role of several specific systems.

Immunofluorescence studies indicate that mainly γG and γM globulins are deposited in association with complement in glomeruli (63). A unique linear pattern of γG globulin localization was observed in several patients without evidence of glomerulonephritis (Figure 5*A*). Three major patterns of deposition of immunoglobulins and complement were found in glomeruli (64). Mesangial deposits were found in a characteristic pattern of irregular, homogeneous strands of protein lying between capillary loops (Figure 5*B*). These were found in patients with clinically active disease and complement depression but with no microscopic evidence of glomerular abnormality or significant proteinuria. The mesangium may serve to clear macromolecular substances, and when it is saturated complexes are deposited along the basement membrane. Certain patients with repeated episodes of hypocomplementemia did not develop proteinuria, and their kidneys showed only mesangial deposits. In other patients, however, minimal mesangial deposits were associated with granular deposits early in the course of the disease, suggesting that certain types of complexes may have an affinity for the glomerular basement membrane. Granular deposits were of two types. In most cases, they were found to be of irregular size and distribution throughout the glomerulus (Figure 5*C*), whereas in several patients with evidence of membranous nephropathy and nephrotic syndrome, a fine homogeneous granular deposition was observed in most glomeruli. In more advanced types of nephritis, coarse lumpy deposits (Figure 5*D*) were found irregularly distributed in the glomerular tufts. Differences were observed in the types of immunoglobulin present in the glomerulus. In certain cases, exclusively γG globulin deposition was observed in the glomerulus, in others a mixture of γG and γM globulins was found, and in a few cases predominantly γM globulin was observed in the glomerulus (55, 64). Selective deposition of subgroups of γG globulin in individual cases has also been demonstrated (65). The implications of γM globulin deposition in the glomerulus will be discussed in a subsequent section. In addition, studies of complement components indicated that two patterns of complement deposition could be demonstrated in renal glomeruli. In one, both $C1_q$ and C3 globulin were present in the glomerulus, and in the second, C3 globulin was demonstrable in the absence of $C1_q$ globulin; both were associated with deposition of immunoglobulins (66). Depression of early-acting components of complement in serum suggests that immune complexes operate via the classical complement pathway (67, 68), although the alternate pathway of complement activation may also be utilized by certain types of complexes. In several studies, the deposition of properdin in glomeruli was found to be minimal in SLE (66, 69), whereas one investigation indicated that significant deposits of properdin occurred (70), lending support to the hypothesis that the alternate pathway is functional in patients with SLE. Serological studies indicate that depression of C3 proactivator and properdin has been found in the sera of patients with active SLE (71), providing further evidence that this pathway may also be functional. Therefore, immunofluorescence studies showing variable mesangial deposition, different patterns of granular deposits, and differences in the types of

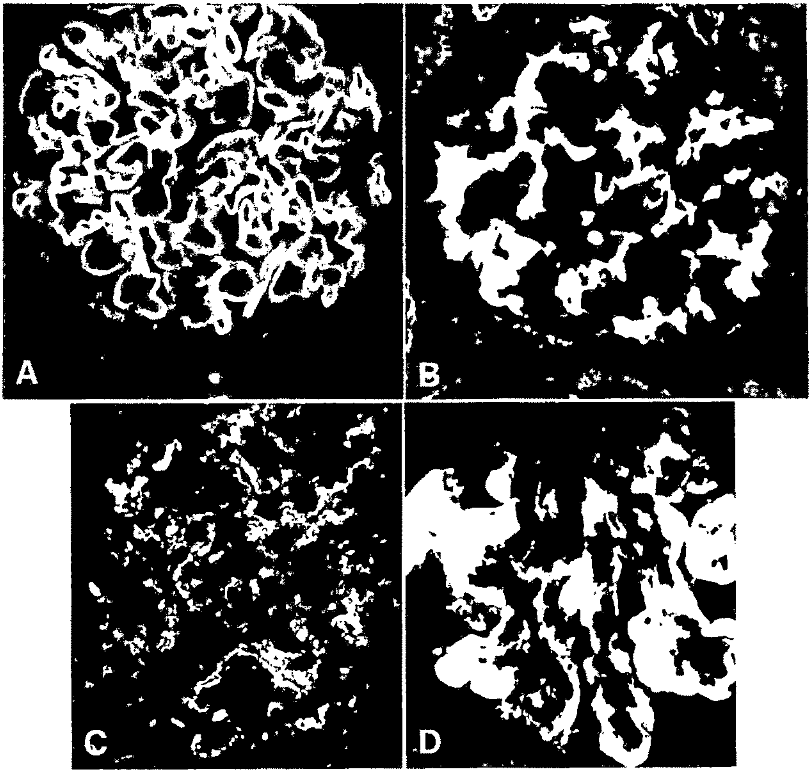


Figure 5 Different patterns of fluorescence in four SLE biopsies. (*A*) Patient D.S.: linear deposits of γG globulin along GBM. (*B*) Patient P.W.: mesangial deposits of γ globulin shown as irregular strandlike areas of fluorescence lying between capillary loops. (*C*) Patient J.B.: granular deposits of γ globulin along GBM. (*D*) Patient M.R.: lumpy deposits of immunoglobulin outlining tufts. All X 250. From reference 64.

immunoglobulins and complement component deposits in renal glomeruli all suggest heterogeneity of immune complex deposition.

The significance of the linear localization of γG globulin in the absence of complement deposition has not been determined. Attempts to characterize these antibodies by glomerular elution have been unsuccessful. A similar linear deposition of immunoglobulin on the basement membrane of the skin (72) has been characterized by elution studies and has been demonstrated to be reactive with cutaneous but not glomerular basement membranes (73, 74). It is possible that these antibodies sensitize the skin and create a susceptibility for cutaneous injury resulting in the local formation of antigen-antibody complexes, which contain DNA (75).

Isolation of glomeruli from kidneys with severe lupus glomerulonephritis and treatment of these glomeruli with acid buffer and deoxyribonuclease indicates that certain types of antibodies are selectively concentrated (76, 77) (Table 4). Anti-SDNA and anti-NDNA antibodies are found in glomerular eluates obtained from most kidneys (49, 76). Treatment of tissue sections of these kidneys with high molarity salt solution and acid buffer has enabled the detection of both NDNA (76) and SDNA (78) antigen with highly purified fluorescein-labeled anti-NDNA antibody and rabbit anti-SDNA antibody. Anti-ribonucleoprotein antibodies were observed in a few eluates (49), although ribonucleoprotein antigen could not be demonstrated in cryostat sections. Anti-DSRNA antibodies were not demonstrable in acid buffer eluates or in eluates obtained from glomeruli treated with ribonuclease. These studies provide supporting data for the glomerular localization of polynucleotide immune complex systems involving NDNA, SDNA, and ribonucleoprotein. In addition, evidence for the participation of gamma-globulin–anti-gamma-globulin complexes was also found. Renal biopsies obtained from patients with circulating cryoglobulins showed significant quantities of glomerular deposition of γM globulin (Table 5). In several of these biopsies, rheumatoid factor activity was demonstrable using fluorescein-labeled aggregregated gamma globulin (49). Direct evidence for the presence of rheumatoid factor was obtained from a patient with a circulating cryoglobulin. A fluorescein-labeled idiotypic antiserum prepared to the isolated cryoglobulin stained glomerular deposits of a renal biopsy obtained from the same patient, indicating that the circulating cryoglobulin was concentrated in glomeruli (55).

Central nervous system disease in lupus appears to be associated with vasculitis involving small blood vessels (79). Necrosis, thrombi, and proliferative changes in arterioles and capillaries have been found to occur with microinfarcts. Involvement of larger vessels in the brain is not prominent, although fibrinoid necrosis of medium-size arteries associated with gamma globulin, complement, and DNA antigen has been found in the renal parenchyma (80). There is evidence suggesting that immune complexes are present in the choroid plexus (81) and in the cerebrospinal fluid (82). These complexes may be responsible for central nervous system injury,

Table 4 Concentration of antibodies in glomerular eluates[a]

	Total Number Studied	Anti NDNA	Anti SDNA	Anti RNA Pr[b]
Acid buffer eluates	9	5[c]	6	3
Deoxyribonuclease eluates	9	6	8	1

[a] From reference 49.

[b] RNA Pr, ribonucleoprotein.

[c] Number of eluates with increased antibody activity per mg of globulin in comparison with serum.

Table 5 Serological and immunofluorescent findings in SLE patients with cryoprecipitins and $C1_q$ precipitin reactions[a]

		Cryoprecipitins			Renal Biopsy			
	mg/100 ml	Rheumatoid Factor	$C1_q$ Precipitation	Pattern of Deposit	γG	γM	RF	Proteinuria g/24 hr
M.R.	36.8	+	++	Granular	3+	3+	2+	4.8
J.M.H.	14.4	+	+++	Granular	3+	2+	Tr	5.7
J.H.	10.0	+	++	Granular	3+	3+	2+	1.4
A.R.[b]	12.0	+	++	Granular	1+	3+	0	1.6
G.G.	2.8	0	++	Mesangial	2+	Tr	NS[c]	0
S.V.	3.6	0	++	Mesangial	3+	0	NS	0.1
D.S.	2.7	0	+	Mesangial	3+	0	NS	0

[a] From reference 55.
[b] With Sjogren's syndrome.
[c] NS – not studied.

although there is a paucity of information concerning the localization of immunoglobulins and complement. One immunofluorescent study of brain tissue from three patients with neurological manifestations did not reveal gamma-globulin localization (79). The role of immune complexes in the pathogenesis of central nervous system injury remains to be clarified by further investigation.

Extensive serologic investigations, epidemiological studies, and electron microscopic examination of tissues from patients with SLE have suggested a variety of etiologic factors contributing to the immunologic abnormalities occurring in patients with SLE. The familial occurrence of SLE has been cited in several studies (83, 84), as well as an increased incidence of antinuclear antibodies in the relatives of patients with SLE (85). A tentative relationship between certain histocompatibility antigens and SLE, which may implicate genetic factors in the etiology of the disease, has been established (86). The naturally occurring form of systemic lupus erythematosus in mice, coupled with the ability to manipulate inbred strains of mice to produce hybrid strains with a higher incidence of renal disease, would suggest the importance of genetic factors in the animal model of this disease (87).

The ingestion of certain drugs such as procainamide (88) and hydralazine (89) has been associated with the presence of antinuclear antibodies and syndromes with similarities to clinical SLE, indicating that certain chemicals, or metabolites of these chemicals, may be responsible for initiating the syndrome of SLE. In view of the known photosensitivity of patients with SLE, the role of ultraviolet (UV) radiation as a possible etiologic agent has been discussed (90). UV-irradiated DNA, a potent immunogen (91), could theoretically be responsible for the breaking of tolerance to native DNA antigen. Neither antibodies to UV-irradiated DNA nor UVDNA antigen have been found in the sera of patients with SLE, although UVDNA has been induced in vivo in man (90), and complexes containing UVDNA have been shown to be capable of inducing glomerulonephritis in rabbits (92).

The role of viral infection as a potential etiologic factor, as an immunogen for certain of the antibodies found in the sera of these patients, and as a contributor to antigen-antibody complex formation has been raised by several observations. Electron microscopic studies have shown that tubuloreticular-like structures are present in the endothelium of glomeruli, skin, and lymphocytes of patients with SLE (93–100). These particles were initially considered to have morphological resemblance to myxovirus particles, but evidence has been presented suggesting that these structures represent a cellular reaction to viral infection rather than nucleoprotein cores (96, 100). The presence of antibodies reactive with double-stranded RNA has been offered as evidence that double-stranded RNA virus is present and serves as an immunogen in SLE patients (33). These antibodies, however, have not been shown to have unique specificity for double-stranded viral RNA, and may well be induced by single- or double-stranded polyribonucleotides of either viral or cellular origin. Titers of antibodies to other viruses, especially myxoviruses, are elevated in the sera of SLE patients (101–103). The present evidence concerning the role of viral infection in systemic lupus erythematosus clearly merits further investigation, but the relevance of viral infection to the etiology and pathogenesis of SLE has not been clarified.

In summary, the immunopathogenesis of SLE has been established and certain immune complex systems have been shown to be operative in the induction of glomerulonephritis. The relative contributions of the specific systems that have been identified remain to be ascertained. These observations have stimulated the use of immunosuppressive therapy in an attempt to decrease the morbidity and mortality of glomerulonephritis, with encouraging results (104). The pathogenesis of the vasculitis and central nervous system disease is less certain, although antigen-antibody complexes have been implicated here as well. A host of etiologic factors, including genetic, viral, chemical, and physical agents, have been suggested, but as yet none has been shown to have a definitive role. Future investigation into the etiology and pathogenesis of SLE may help to give us further insight into the general mechanisms of immunologically induced tissue injury in man.

Literature Cited

1. Robbins, W. C., Holman, H. R., Deicher, H., Kunkel, H. G. 1957. *Proc. Soc. Exp. Biol. Med.* 96:575–79
2. Seligmann, M. 1957. *C. R. Acad. Sci.* 245:243–45
3. Deicher, H. R., Holman, H. R., Kunkel, H. G. 1959. *J. Exp. Med.* 109:97–114
4. Arana, R., Seligmann, M. 1967. *J. Clin. Invest.* 46:1867–82
5. Koffler, D., Carr, R. I., Agnello, V., Feizi, T., Kunkel, H. G. 1969. *Science* 166:1648–49
6. Koffler, D., Carr, R. I., Agnello, V., Thoburn, R., Kunkel, H. G. 1971. *J. Exp. Med.* 134:294–312
7. Jokinen, E. J., Julkunen, H. 1965. *Ann. Rheum. Dis.* 24:477–80
8. Wold, R. T., Young, F. E., Tan, E. M. 1968. *Science* 161:806–7
9. Pincus, T., Schur, P. H., Rose, J. A. 1969. *N. Engl. J. Med.* 281:701–5
10. Carr, R. I., Koffler, D., Agnello, V., Kunkel, H. G. 1969. *Clin. Exp. Immunol.* 4:527–36
11. Ginsberg, B., Keiser, H. 1973. *Arthritis Rheum.* 16:199–207
12. Epstein, W. V., Tan, M., Easterbrook, M. 1971. *N. Engl. J. Med.* 285:1502–6
13. Koffler, D. et al. Unpublished observation
14. Tan, E. M., Schur, P. H., Carr, R. I., Kunkel, H. G. 1966. *J. Clin. Invest.* 45:1732–61
15. Cohen, S. A., Hughes, G. R. V., Noel, G. L., Christian, C. L. 1971. *Clin. Exp. Immunol.* 8:551–61
16. Barbu, E., Seligmann, M., Joly, M. 1960. *Ann. Inst. Pasteur* 99:695–707
17. Stollar, D., Levine, L. 1961. *J. Immunol.* 87:477–84
18. Stollar, D., Levine, L., Lehrer, H. I., Van Vunakis, H. 1962. *Proc. Nat. Acad. Sci.* 48:874–80
19. Stollar, D., Levine, L. 1963. *Arch. Biochem.* 101:417–22
20. Holman, H. R., Kunkel, H. G. 1957. *Science* 126:162–63
21. Holman, H., Deicher, H. R. 1959. *J. Clin. Invest.* 38:2059–72
22. Tan, E. M. 1967. *J. Clin. Invest.* 46:735–45
23. Robitaille, P., Tan, E. M. 1973. *J. Clin. Invest.* 52:316–23
24. Tan, E. M., Kunkel, H. G. 1966. *J. Immunol.* 96:464–71
25. Holman, H. R. 1965. *Ann. N. Y. Acad. Sci.* 124:800–6
26. Sharp, G. C. et al 1971. *J. Clin. Invest.* 50:350–59
27. Mattioli, M., Reichlin, M. 1971. *J. Immunol.* 107:1281–90
28. Mattioli, M., Reichlin, M. 1973. *J. Immunol.* 110:1318–24
29. Schur, P. H., Moroz, L., Kunkel, H. G. 1967. *Immunochemistry* 4:447–53
30. Asherson, G. L. 1959. *Brit. J. Exp. Pathol.* 40:209–15
31. Deicher, H. R. G., Holman, H. R., Kunkel, H. G. 1960. *Arthritis Rheum.* 3:1–15
32. Schur, P. H., Stollar, D., Steinberg, A. D., Talal, N. 1971. *Arthritis Rheum.* 14:342–47
33. Talal, N., Steinberg, A. D., Daley, G. 1971. *J. Clin. Invest.* 50:1248–52

34. Sylvester, R. A., Attias, M., Talal, N., Tuffanelli, D. L. 1973. *Arthritis Rheum.* 16:383–87
35. Terasaki, P. I., Mottironi, V. D., Barnett, E. V. 1970. *N. Engl. J. Med.* 283: 724–28
36. Stastny, P., Ziff, M. M. 1971. *Clin. Exp. Immunol.* 8:543–50
37. Mittal, K. K., Rossen, R. D., Sharp, J. T., Livsky, M. D., Butler, W. T. 1970. *Nature (London)* 225:1255–56
38. Williams, R. C. Jr., Emmons, J. D., Yunis, E. J. 1971. *J. Clin. Invest.* 50: 1514–24
39. Wernet, P., Kunkel, H. G. 1973. *Fed. Proc.* 32:975 Abs(4248)
40. Wernet, P., Fotino, M., Thoburn, R., Moore, A., Kunkel, H. G. 1973. *Arthritis Rheum.* 16:137 (abs)
41. Lies, R. B., Messner, R. P., Williams, R. C. Jr. 1973. *Arthritis Rheum.* 16: 369–75
42. Wernet, P., Kunkel, H. G. 1973. *J. Exp. Med.* 138:1021–46
43. Hedberg, H., Kallen, B., Low, B., Nilsson, O. 1971. *Clin. Exp. Immunol.* 9: 201–7
44. Abe, T., Homma, M. 1971. *Acta Rheumatol. Scand.* 17:35–46
45. Block, S. R., Gibbs, C. B., Stevens, M. B., Shulman, L. E. 1968. *Ann. Rheum. Dis.* 27:311–18
46. Horwitz, D. A. 1972. *Arthritis Rheum.* 15:353–59
47. Hahn, B. H., Babgy, M. K., Osterland, C. K. 1973. *Am. J. Med.* 55:25–31
48. Schur, P. H., Sandson, J. 1968. *N. Engl. J. Med.* 278:533–38
49. Koffler, D., Agnello, V., Thoburn, R., Kunkel, H. G. 1971. *J. Exp. Med.* 134:169–75s
50. Koffler, D., Agnello, V., Winchester, R., Kunkel, H. G. 1973. *J. Clin. Invest.* 52:198–204
51. Christian, C. L., Hatfield, W. B., Chase, P. H. 1963. *J. Clin. Invest.* 42:823–29
52. Hanauer, L. B., Christian, C. L. 1967. *J. Clin. Invest.* 46:400–3
53. Koffler, D., Agnello, V., Thoburn, R., Kunkel, H. G. 1970. *Immunopathol. Int. Symp. 6th,* ed. P. A. Miescher, 336–45. Basel, Switz.: Schwabe
54. Agnello, V., Koffler, D., Eisenberg, J. W., Winchester, R. J., Kunkel, H. G. 1971. *J. Exp. Med.* 134: 228–41s
55. Agnello, V., Koffler, D., Kunkel, H. G. 1973. *Kidney Int.* 3:90–99
56. Davis, G. L. Jr., Davis, J. S. IV. 1973. *Arthritis Rheum.* 16:52–58
57. Harbeck, R. J., Bardana, E. J., Kohler, P. F., Carr, R. I. 1973. *J. Clin. Invest.* 52:789–95
58. Carr, R. I. 1969. *DNA antibodies and DNA in serum.* PhD thesis. The Rockefeller University, New York
59. Hughes, G. R. V., Cohen, S. A., Lightfoot, R. W. Jr., Meltzer, J. I., Christian, C. L. 1971. *Arthritis Rheum.* 14: 259–66
60. Koffler, D. et al. Unpublished results
61. Thoburn, R. et al. Unpublished results
62. Agnello, V., Winchester, R. J., Kunkel, H. G. 1970. *Immunology* 19:909–19
63. Paronetto, F., Koffler, D. 1965. *J. Clin. Invest.* 44:1657–64
64. Koffler, D., Agnello, V., Carr, R. I., Kunkel, H. G. 1969. *Am. J. Pathol.* 56:305–12
65. Lewis, E. J., Busch, G. J., Schur, P. H. 1970. *J. Clin. Invest.* 49:1103–13
66. Koffler, D., Agnello, V., Kunkel, H. G. *Am. J. Pathol.* In press
67. Kohler, P. F., Ten Bensel, R. 1969. *Clin. Exp. Immunol.* 4:191–202
68. Morse, J. H., Müller-Eberhard, H. J., Kunkel, H. G. 1962. *Bull. N. Y. Acad. Med.* 38:641–51
69. Michael, A. F., Westberg, N. G., Fish, A. J., Vernier, R. L. 1971. *J. Exp. Med.* 134:208–27s
70. Rothfield, N., Ross, H. A., Minta, J. O., Lepow, I. H. 1972. *N. Engl. J. Med.* 287:681–85
71. McLean, R. H., Michael, A. F. 1973. *J. Clin. Invest.* 52:634–44
72. Tuffanelli, D. L., Kay, D., Fukuyama, K. 1969. *Arch. Dermatol.* 99:652–62
73. Landry, M., Sams, W. M. Jr. 1972. *Lancet* 1:821–22
74. Quismorio, F. P., Wierchowiecki, M., Friou, G. J. 1973. *Arthritis Rheum.* 16:30 (abs)
75. Tan, E. M., Kunkel, H. G. 1966. *Arthritis Rheum.* 9:37–46
76. Koffler, D., Schur, P. H., Kunkel, H. G. 1967. *J. Exp. Med.* 126: 607–23
77. Krishman, C., Kaplan, M. H. 1967. *J. Clin. Invest.* 46:569–79
78. Andres, G. A. et al 1970. *J. Clin. Invest.* 49:2106–18
79. Johnson, R. T., Richardson, E. P. 1968. *Medicine* 47:337–69
80. Koffler, D., Schur, P. H., Kunkel, H. G., Graf, M. 1967. *Immunopathol. Int. Symp. 5th,* ed. P. A. Miescher, P. Grabar, 70–79. Basel, Switz.: Schwabe

81. Atkins, J. K., Quismorio, F. P., Friou, G. J. 1971. *Arthritis Rheum.* 14:148 (abs)
82. Harbeck, R. J., Hoffman, A. A., Carr, R. I. 1973. *Arthritis Rheum.* 16:27 (abs)
83. Brunjes, S., Zike, K., Julian, R. 1961. *Am. J. Med.* 30:529–36
84. Arnett, F. C., Bias, W. B., Shulman, L. E. 1972. *Arthritis Rheum.* 15:102 (abs)
85. Koffler, D., Agnello, V., Kunkel, H. G. Unpublished results
86. Grumet, F. C., Coukell, A., Bodmer, J. G., Bodmer, W. F., McDevitt, H. O. 1971. *N. Engl. J. Med.* 285: 193–96
87. Howie, J. B., Helyer, B. J. 1968. *Advances in Immunology,* ed. F. J. Dixon Jr., H. G. Kunkel. 9:215–66. New York: Academic
88. Dubois, E. L. 1969. *Medicine* 48: 217–28
89. Alarcon-Segovia, D., Wakin, K. G., Worthington, J. W., Ward, I. E. 1967. *Medicine* 46:1–33
90. Tan, E. M., Stoughton, R. B. 1969. *J. Invest. Dermatol.* 52:537–42
91. Tan, E. M. 1968. *Science* 161:1353–54
92. Natali, P. G., Tan, E. M. 1972. *J. Clin. Invest.* 51:345–55
93. Györkey, F., Sinkovics, J. G., Min, K. W., Györkey, P. 1969. *N. Engl. J. Med.* 280:333
94 Kawano, K., Miller, L., Kimmelstiel, P. 1969. *N. Engl. J. Med.* 281:1228–29
95 Grausz, H., Earley, L. E., Stephens, B. G., Lee, J., Hopper, J. Jr. 1970. *N. Engl. J. Med.* 283:506–11
96 Pincus, T., Blacklow, N. R., Grimley, P. M., Bellanti, J. A. 1970. *Lancet* 2: 1058–61
97 Garancis, J. C., Komorowski, R. A., Bernhard, G. C., Straumfjord, J. V. 1971. *Am. J. Pathol.* 64:1–12
98. Hurd, E. R., Eigenbrodt, E., Worthen, H., Strunk, S. S., Ziff, M. 1971. *Arthritis Rheum.* 14:539–50
99. Györkey, F., Sinkovics, J. G., Min, K. W., Györkey, P. 1972. *Am. J. Med.* 53:148–58
100. Grimley, P. M., Decker, J. L., Michelitch, H. J., Frantz, M. M. 1973. *Arthritis Rheum.* 16:313–23
101. Phillips, P. E., Christian, C. L. 1970. *Science* 168:982–84
102. Hollinger, F. B., Sharp, J. T., Lidsky, M. D., Rawls, W. E. 1971. *Arthritis Rheum.* 14:1–14
103. Evans, A. S., Rothfield, N. F., Niederman, J. C. 1971. *Lancet* 1:167–68
104. Ginzler, E., Sharon, E., Diamond, H., Kaplan, D. 1973. *Arthritis Rheum.* 16:43 (abs)

Copyright 1974. All rights reserved

IMMUNO-PREVENTION OF Rh HEMOLYTIC DISEASE OF THE NEWBORN

❖7105

R. B. McConnell, M.D., F.R.C.P. and J. C. Woodrow, M.D., F.R.C.P.
Department of Medicine, University of Liverpool, Liverpool, England

When this subject was last reviewed in this publication (1) it had just been established in a small, carefully controlled trial (2) that the incidence of maternal Rh immunization could be markedly reduced by injecting anti-D immunoglobulin into mothers soon after their delivery of an Rh-positive baby. Thus the technical knowledge had become available to prevent most instances of a disease that affected one in 250 live births in European communities. This knowledge has been seized upon, anti-D immunoglobulin has been produced in large quantities by various governmental health services and commercial enterprises, and now, eight years later, large scale application of the technique to Rh-negative women after delivery is being carried out. Already there is evidence of the need for fewer exchange transfusions and the next decade should see the reduction of Rh hemolytic disease of the newborn to the status of a rare disease.

Not all problems, however, are solved. Where the usually recommended prophylactic technique is being applied there is still a 1% Rh-immunization rate, representing a 1 in 10 failure of protection that is largely due to intrapregnancy immunization. There is also the problem of Rh immunization caused by abortions, and the steps needed to deal with these practical problems are discussed in this contribution. Also discussed is the theoretical problem of how the injection of anti-D immunoglobulin succeeds in preventing immunization. An understanding of the exact immunological mechanism involved would be an important contribution to the wider fields of tumor and transplant immunology.

NATURAL HISTORY OF Rh IMMUNIZATION

Interest in the process by which Rh-negative mothers become immunized by Rh-positive erythrocytes has continued in recent years because detailed and accurate information is fundamental to the optimal application of Rh prophylaxis by the administration of anti-D IgG. The timing, quantity, and exact indications for the administration of anti-D and assessment of the results obtained depend on an understanding of this process.

The two main techniques used in studying this process are the acid elution method for detecting fetal cells in maternal blood (3) and tests for serum Rh antibodies. The problems of applying the fetal cell technique have been critically reviewed (4, 5). The detection of small numbers of fetal cells is by no means a very reliable procedure as is evident from the great variations in the reports of the incidence of transplacental hemorrhage during pregnancy and in relation to abortion. Up to 2% of normal individuals in several European countries show increased levels of Hb F, and in the first trimester of pregnancy a proportion of mothers also show an appreciable rise. The characteristic appearance of the eluted and stained films in such people was reported several years ago (6) and the point made that recognition and counting of fetal cells was difficult and sometimes impossible in their blood. Increasingly sensitive methods are being used in many laboratories for testing for antibodies, and an antibody detected in one laboratory might be missed in another.

The General Picture of Rh Immunization

Rh antibody may appear for the first time during a particular pregnancy, including the first, either as a primary or secondary response, the latter depending on a previous stimulus from an Rh-positive pregnancy, an abortion, or a transfusion or injection of Rh-positive blood. The incidence of antibodies appearing in a first pregnancy varies from 0.5 – ∾ 2% when the pregnancy is ABO-compatible (antibody appearing in a first ABO-incompatible pregnancy must be exceedingly rare). Antibody present early in the first pregnancy must have been the result of a previous stimulus and will have been present prior to the onset of pregnancy. In most instances antibody does not appear until the third trimester, and it is during this period that there is a definite incidence of transplacental hemorrhage. It is likely that some of these cases represent a primary antibody response. Repeated reference has been made in the literature to a diminished potentiality for producing a primary antibody response during pregnancy, but the only direct evidence refutes this concept (7, 8).

The incidence and degree of transplacental hemorrhage increases markedly as the result of labor. Approximately 50% of mothers show fetal cells postpartum when the pregnancy is ABO-compatible. In most instances the volume involved is small, but in about 0.2% it is of the order of 25–30 ml or more (Figure 1). It is very likely that some mothers are stimulated to produce antibody by extremely small amounts of Rh-positive blood. Because of the very variable sensitivity of Rh-negative individuals to Rh-positive red cells, the concept of a minimum immunizing dose can only be statistical. A very small volume of red cells, while failing to immunize most Rh-negative individuals, may yet result in antibodies in a very few.

Six months after delivery of an ABO-compatible Rh-positive first baby approximately 8% of mothers show anti-D, the incidence being about one tenth of this when the pregnancy is ABO-incompatible. It is of interest that the reported incidence after ABO-compatible pregnancies is appreciably lower in some countries, e.g. 4.9% in

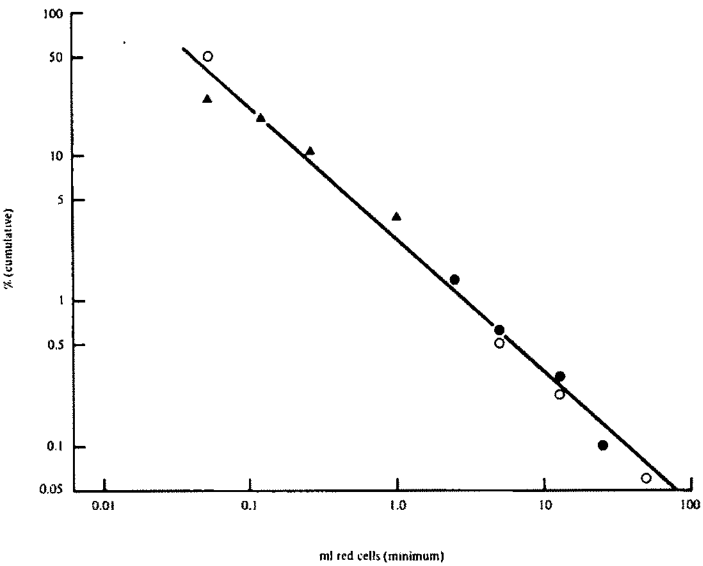


Figure 1 Estimates of the extent of transplacental hemorrhage at the time of delivery in three different series: ▲ = Woodrow & Donohoe (55); ○ = Borst-Eilers (56); ● = Poulain & Huchet (57). Only women with ABO-compatible infants were included in the first series; in the other two series, the cases were unselected. Each point indicates the percentage of women found to have *x* ml or more fetal red cells in their circulation immediately after delivery. Reproduced with the permission of Dr. P.L. Mollison (69).

West Germany (9) and 4.3% in Finland (10). Approximately 3% of mothers showing no fetal cells immediately after delivery of an ABO-compatible infant develop anti-D. In some of these there is a delay in the appearance of fetal cells for several days (11) and in others there has probably been a transplacental hemorrhage in the weeks prior to delivery.

About 8% of mothers develop anti-D during the second Rh-positive pregnancy when the first was ABO-compatible, and most of these must represent secondary responses: priming has occurred as a result of the first pregnancy but detectable antibody has not resulted. In some, however, there may have been a primary response during the second pregnancy, and patterns of antibody development suggestive of these two types of response have been documented (12). Antibody appearing during a second ABO-incompatible pregnancy would point strongly to a secondary response.

Abortion and Rh Immunization

It is not easy to estimate accurately the quantitative risk of immunization involved in spontaneous and therapeutic abortion. An ideal study would involve a prospective survey of Rh-negative women with a first-pregnancy abortion, testing for Rh antibody at the end of a subsequent full-term pregnancy and comparing this incidence with that found at the end of such pregnancies where there had been no prior abortion. Retrospective studies involve a danger of biased sampling as mothers with antibody in a first full-term pregnancy may well be questioned more closely about prior abortion than those without antibody.

Many studies of the incidence of transplacental hemorrhage in relation to abortion have been carried out and a notable feature is the extreme divergence of results (13). This is almost certainly mainly due to the difficulties in applying the acid elution technique. The results do, however, suggest that induced abortion is associated with the more frequent occurrence of sizable transplacental hemorrhage, e.g. 0.2 ml or more of fetal blood is reported in approximately 5% of cases.

From the point of view of Rh immunization, an abortion may (*a*) have no effect, (*b*) result in a primary antibody response, (*c*) produce priming without detectable antibody, the latter appearing in a subsequent Rh-positive pregnancy, or (*d*) induce antibody as a secondary response. Table 1 summarizes the results of several clinical studies.

The differences in the incidences of antibody probably reflect differences in selection of women for study, in the methods used for antibody detection, and chance variation. The evidence points to induced abortion being a more potent cause of immunization than spontaneous abortion and, taking into account that in most studies 40% of the abortions were Rh-negative, a surprisingly high incidence of antibody has been found. Appreciable numbers of well-documented cases of immunization by a first-pregnancy spontaneous abortion, especially with a gestational age of 14 weeks or more, have been reported, and undoubtedly some risk is involved. Before ten weeks' gestation, spontaneous abortion is associated with a very small risk of immunization.

Other Obstetric Complications

During the antenatal period certain events are of potential importance in regard to transplacental hemorrhage and therefore to the risk of immunization. External version, abdominal trauma, antepartum hemorrhage, and amniocentesis performed for the assessment of possible or known hemolytic disease and earlier in pregnancy for genetic counseling, are all associated with an increased risk. Complicated deliveries have been repeatedly documented as liable to cause transplacental hemorrhage (14).

MECHANISM OF IMMUNOSUPPRESSION BY ANTIBODY

The phenomenon of antibody-mediated immunosuppression has been observed in different experimental and clinical contexts over the past seventy three years (15). Because of the complexity of the specific antibody response it would be naive to expect that the mechanism of immunosuppression should be the same in all situations, or that there might not be more than one mechanism operating at the same time. Our ideas on this question cannot be dissociated from current concepts in regard to the normal processes of the antibody response and, as these are at present still fragmentary, it is not surprising that definitive answers are not possible.

Table 1 Rh immunization and abortion

				Developing Antibody			
Authors	Type of Abortion	Gravidity	Number	Number	Percent	Timing of Antibody Tests	Immunizing Abortions
Freda et al (58)	Spontaneous	1	162	7	4.3	First half of subsequent Rh+ pregnancy	6 spontaneous (3 D & C) (? gestational age) 1 ectopic
Queenan et al (59)	Spontaneous	1	32	1	3.1	During subsequent pregnancy	16 weeks: D & C
Visscher & Visscher (60)	Spontaneous	Variable	38	0	–	6 months later	–
Queenan et al (61)	Induced	Variable	25	1	4.0	4 months later	Suction curretage in multip. at 9 weeks
Matthews et al (62)	Spontaneous + Induced	Variable	96	1	1.0	6 months later	16 weeks in 4th pregnancy: vaginal termination
Jorgensen (63)	Spontaneous + Induced	Variable	30	3	10.0	6–12 months later	1 primip: vaginal termination 2 multips: ? type
Wallace & MacDougall (64)	Unspecified	1	370	4	1.1	During subsequent Rh+ pregnancy	?
Murray & Barron (65)	Spontaneous + Induced	1	48	3	6.3	After variable number of months	All induced 10–18 weeks: method varied
		>1	48	6	12.5		
Ortho-associated Study (66)	Spontaneous + Induced	Variable	115	4	3.5	?	All induced 10–18 weeks: method varied
Gavin (67)	Spontaneous + Induced	Variable	36	2	5.6	3–4 months later	Induced 11 weeks: both primips

When ABO-compatible Rh-positive red cells enter the circulation of an Rh-negative individual the older cells are gradually taken up by macrophages in the spleen and, to some extent, in lymphoid tissues elsewhere. It is likely that most of the red cell membrane is degraded and rendered nonimmunogenic, probably within intracellular lysosomal vesicles (16). Some fragments of membrane probably become attached to the surface of macrophages, and it is likely that these are the effective agents in the induction of the next stages of the immune response (17). Recognition of antigenic determinants by thymus-derived lymphocytes (T-cells) and reaction with specific receptors on bone-marrow-derived lymphocytes (B-cells) are the two basic elements of the immune response. The exact mechanism by which T-cells and B-cells cooperate in their response to antigen is at present the subject of intensive study. There is evidence that the carrier part of the antigen is recognized by and interacts with the IgM-type of receptor on the surface of T-cells, this being followed by release of the complex thus formed. The complex is probably then arranged in suitable form, possibly on the surface of a macrophage, so that an effective stimulation of the specific B-cells can occur via interaction of the haptenic part of the antigen with Ig receptors on these lymphocytes. The B-cell is thus stimulated to divide and differentiate with the consequent secretion of antibody.

Specific antibody could lead to a suppression of this process at several possible points. As the initiation of the immune process depends on the localization of antigen at specialized functional sites where the appropriate macrophages and antigen sensitive cells are present, deviation of the red cell or other immunogen to some other site could prevent any possibility of an immune response. ABO-incompatibility might operate, at least in part, in this way because the majority of red cells in this situation are cleared to the liver where complete degradative phagocytosis occurs and there is no possibility of an immune response. However, anti-D IgG clears most of any circulating Rh-positive red cells to the spleen and other immunocompetent tissues, and so any deviation of antigen would have to be within these tissues.

It has been shown that mouse peritoneal macrophages that have engulfed sheep red cells (SRBC) can induce an antibody response after transfer to a normal recipient, but this response is much reduced if the donor mouse has been passively immunized with anti-SRBC serum (18, 19). It was concluded that the antibody must have increased the rate of antigen degradation by macrophages. However, when the rate of antigen degradation within macrophages was directly measured, SRBC coated with 7S antibody were degraded no more rapidly than uncoated cells (10). In spite of the fact that the mean number of red cells phagocytized was considerably increased by antibody, there was marked depression of the antibody response after transfer to normal recipients. It is of interest that while 19S antibody increased the rate of intracellular degradation, there was a greater antibody response after transfer than was found in the case of 7S antibody. It is not easy to reconcile these findings at present unless it is supposed that some antibody remained attached to the transferred macrophages. That antibody-mediated immunosuppression acted through some function of the macrophage was supported by the in vitro experiments of Pierce (21) in which anti-SRBC antibody suppressed the plaque-forming cell (PFC) response of mouse spleen cells cultured with SRBC. Incubation of antibody with macrophages before their addition to the system depressed the PFC response but preincubation with lymphocytes before addition had no such effect.

One approach to the question of deviation or degradation of the whole cell as the way in which immunosuppression occurs is to examine the specificity of immunosuppression in relation to the specificity of the antibody used. Particulate antigens, such as a red cell, possess many surface determinants, and if antibody to one determinant suppresses the active antibody response to it, it should also, if degradation of the whole red cell were involved, result in lack of antibody responses to the other determinants. The evidence is contradictory, some experimental studies favoring specificity of immunosuppression in this situation (22–24) while others have found nonspecific immunosuppression (25–27). It is likely that the amount, class, and biological activity, including the ability to fix complement, of the antibody used, as well as the nature of the antigen system involved, are important variables that determine the outcome of this type of experiment. In the Rh situation there are no published data on the specificity of immunosuppression, and indeed it will require a very large series of treated mothers to show whether giving Rh antibody leads to a significantly lower incidence of non-Rh antibodies than is found in untreated controls. Many preparations of anti-D include antibodies with other Rh specificities and sometimes non-Rh specificities, and this must of course be taken into account. An attempt to answer this question by experimental studies on human volunteers using the Rh and Kell systems is currently being conducted in Liverpool. D+K+ red cells are given to D–K– recipients and cleared to the spleen with IgG anti-K. It remains to be seen whether the expected incidence of anti-D occurs.

Antibody could lead to immunosuppression by preventing the interactions between antigens at the surface of macrophages and the receptors present on T- and B-cells by competitively combining with antigenic determinants. It has been shown that the higher the affinity of the antibody, the greater the immunosuppressive effect (28), and this is at the least consistent with the concept that passively introduced antibody is competing for antigenic determinants with the receptors of antigen-sensitive cells. The finding of Siskind et al (29) that partial suppression by passive antibody led to the production of antibody with a higher average affinity than would normally have occurred suggests that immunosuppression is occurring by competitive inhibition of antigen-sensitive lymphocytes with receptors by varying combining power. One argument that has been used against this mechanism of immunosuppression is that antibody may be immunosuppressive in amounts that could only combine with a small proportion of available antigen sites; for instance, Mollison & Hughes-Jones (30) showed that anti-D in amounts sufficient to occupy only 10% of the antigen sites of injected red cells could still prevent immunization. However, only a small proportion of the total antigen ends up at the critical sites on the surface of macrophages, and it is likely that it is only these antigenic determinants that need to be blocked.

Several immunosuppressive studies using the $F(ab')_2$ fragment of antibody have suggested that it is this part of the IgG molecule with the antibody combining sites that is important (31, 32). The necessity of an intact Fc part of the molecule has, however, been supported by other work (33).

In those situations when more than one determinant is present on the same particulate antigen and antibody specifically suppresses response to one but not other determinants, the hypothesis of competitive blocking of antigen determinants is favored.

A possible suppressive effect of passive antibody on the antigen-sensitive lymphocytes was put forward by Rowley & Fitch (34). This has received some support from the in vitro studies of Feldmann & Diener (35) who have suggested that in some circumstances a lattice of antigen combined with antibody is built up on the surface of antigen-sensitive lymphocytes, rendering the cells nonreactive to antigen. This appears, however, to require a critical level of antibody in relation to antigen, and the exact relevance of this type of tolerance induction to the in vivo situation is uncertain. It might play some part in the Rh situation but any tolerant state induced must be transient.

RESULT OF CLINICAL TRIALS

By 1962 experimental work had shown that it was possible to protect Rh-negative volunteers from the immunization caused by injected Rh-positive blood (36–38). At the same time it became apparent that in the majority of cases the immunizing transplacental hermorrhage took place at or near delivery and therefore clinical trials were started.

Controlled Trials

In the England and Baltimore trial primiparae at high risk were selected by studying post-delivery maternal blood for fetal cells and including only those mothers showing evidence of a transplacental hemorrhage of at least 0.2 ml fetal blood. Six months after delivery 38 of 176 untreated control women had developed anti-D while only 1 of 173 treated women had antibodies. At the end of a second Rh-positive pregnancy 20 of 65 controls and 2 of 88 treated women had antibodies (39). From this result it was obvious that the technique works but that it is not 100% successful in preventing immunization.

In a New York clinical trial all Rh-negative women delivered of an ABO-compatible Rh-positive baby were included. Six months after delivery 102 of 1476 untreated women and only 6 of 3389 treated women had developed anti-D. At the end of a subsequent pregnancy 23 of 178 controls and 5 of 395 treated women had become immunized (40).

A third trial with controls was in Germany. Here 89 of 2330 controls had anti-D six months after delivery, while 15 of 3117 women given 200 μg anti-D IgG became actively immunized. After a subsequent Rh-positive pregnancy, 29 of 373 controls and 0 of 138 treated had antibodies (41). Figures from Holland were 17 of 329 controls and 3 of 333 treated women 6 months after delivery (42).

In Liverpool a second lower risk trial was carried out in which alternate women with less than 0.2 ml fetal blood in their circulation after delivery were given 200 μg anti-D. Of the 362 controls 13 developed anti-D within 6 months but none of the 352 treated had antibodies at this stage. By the end of a subsequent Rh-positive pregnancy 13 of 127 controls and 3 of 128 treated women were immunized (43). These data are summarized in Table 2. It can be seen that:

(*a*) The immunization rate in untreated control mothers 6 months after delivery of an Rh-positive baby is between 4 and 7%, and by the end of a subsequent Rh-positive pregnancy it has risen to between 8 and 13%. (The difference is largely accounted for by women who are "primed" by the first baby but who do not develop overt antibodies until they receive a boost from Rh-positive cells during the second pregnancy.)

(*b*) Injection of anti-D IgG soon after delivery results in a dramatic reduction in the immunization rate not only 6 months later but at the end of the next pregnancy. Thus, the anti-D is effectively protecting and not merely suppressing overt immunization.

(*c*) The protection gained by the treatment is not 100% effective.

Other Large Trials

Much valuable data has been collected in other clinical trials. For instance, in 5693 primiparae treated in England and Wales National Health Service hospitals there were 26 failures (0.46%) who had antibodies at six months (44). Of the 4581 in whom the Kleihauer score was known there were 20 failures, 10 in 4458 with small and 10 in 123 with large (over 5 ml) transplacental hemorrhages.

In Australia 21 of 3307 treated women (0.64%) had antibodies at 6 months and 13 of 1218 (1.08%) were immunized by the end of the next pregnancy (45).

Eklund & Nevanlinna (10) have reported the results of the Finnish preventive program comparing their 14,980 treated women with 1707 delivered before the program started in 1969. They had found a 4.3% postpartum immunization rate in the untreated women who had ABO-compatible babies. The rate in 12,703 treated women was 0.13%. Of the 17 who became immunized, 12 were ABO-compatible with the baby and 5 ABO-incompatible. Subsequent Rh-positive babies had been born to 1027 treated women and antibodies appeared during the subsequent pregnancy in 10 of them, 3 before the 30th week and 2 after the 37th week of gestation. The authors interpreted this late appearance of antibodies as indicating that not all these mothers were primed by the first baby, but some had been primarily immunized during the subsequent pregnancy and were, therefore, not failures of the prophylactic injection at delivery of the first baby.

Reasons for Failure of Prophylaxis

As pointed out, injection of anti-D after delivery does not give 100% protection. There are always a few (about 1%) women who become immunized in spite of anti-D IgG administration in the usually recommended dose. As the natural immunization rate is about 10%, this is a failure rate of 1 in 10.

There are probably two main reasons for these failures. A few of them, perhaps 1 in 10, may be due to massive transplacental hemorrhage, which is more than the normal dose of immunoglobulin can cope with. To eliminate failures due to this cause either the routine dose of immunoglobulin would have to be greatly increased

Table 2 Results of controlled clinical trials of the efficacy of anti-D injected after delivery in the prevention of Rh immunization

Trial		Six months after delivery			After subsequent Rh-positive pregnancy		
		Total	Immunized	% Immunized	Total	Immunized	% Immunized
High risk (39)	Controls	176	38	21.6	65	20	30.7
	Treated	173	1	0.6	88	2	2.2
Low risk (39)	Controls	362	13	3.6	127	13	10.2
	Treated	353	0	0	128	3	2.3
New York (68)	Controls	1476	102	6.9	175	23	14.3
	Treated	3389	6	0.2	395	5	1.2
Germany (41)	Controls	2330	89	3.8	373	29	7.8
	Treated	3117	15	0.4	138	0	0
Holland (42)	Controls	329	17	5.0			
	Treated	333	3	0.9			

or some technique applied to detect the women who have received a massive fetal cell transfusion. The ideal would be to examine by the Kleihauer technique every maternal blood after delivery, but this may be practical only in some centers or in a maternity service that most countries could not afford. Mollison (46) has discussed this problem. Difficulty will always arise with the women who have a high Hb F level and who give a graded Kleihauer picture (6). In these women a mixed agglutination technique (47) is needed to tell whether the Hb F-containing cells are Rh-positive (i.e. fetal) or Rh-negative (maternal).

If a baby is born anemic the mother's blood should be examined by the Kleihauer technique, and if this confirms a massive feto-maternal bleed she should be given a dose of at least 20 μg anti-D IgG for each estimated ml of fetal blood. If necessary, more should be given after 24 to 48 hr until no fetal cells can be detected, as, whatever the exact mechanism involved, protection has been found to correlate with rapid clearance of Rh-positive cells. As an alternative to the Kleihauer technique, Gorman (38) suggests that the very large numbers of Rh-positive cells in the Rh-negative mother's blood could be detected by a one-stage agglutination technique giving a positive "D^u test." Even this may not be practicable throughout a large population and, as it would not detect moderately large fetal bleeds (48), it seems that the alternatives are to accept a small number of Rh immunizations (about 0.1% of all Rh-negative women at risk), or test all women after delivery by the Kleihauer technique.

The second, and much more important, reason for the failure of the routine prophylaxis technique is that the women were already in a state of immunization at the time of delivery, even though overt antibodies were not detected. The evidence in favor of this explanation is partly that the Kleihauer score was nil or very low in many of these women. As described earlier, examination of maternal blood throughout pregnancy has often revealed fetal cells from the end of the second trimester onwards, and sizable fetal hemorrhages are especially probable after external version or other trauma of the placenta.

Though Rh hemolytic disease is rare in a first born baby, the concept of intrapregnancy immunization was put forward with particular emphasis by Cohen & Zuelzer (49). Chown and his group in Winnipeg have made the major contribution to the present trend towards antepartum therapy (50).

Antenatal Therapy

Even though most Rh immunizations can be attributed to transplacental passage of fetal red cells during labor (or in the preceding week or two), there are a few that seem to be due to fetal bleeding occurring earlier in pregnancy, especially in the third trimester. Antibodies found at delivery of primigravidae may be associated with previous abortion but, as described earlier, there is no doubt that in some instances transplacental hemorrhage during the pregnancy results in antibodies being found late in pregnancy or within three days of delivery. In addition, as described in the previous section, there is strong evidence that some women with no detectable antibodies at delivery are in fact immunized.

Injections of anti-D soon after delivery cannot be expected to help women who are already immunized, and, if Rh immunization is to be eradicated completely, some additional prophylactic measure will be needed. As early as 1967 Zipursky & Israels (51) reported that anti-D could be given to women during pregnancy without apparent harm to their Rh-positive fetuses. At present 260 μg anti-D are being injected intramuscularly at 28 and 34 weeks gestation, and adequate levels of antibody are maintained through the third trimester. Demonstrable feto-maternal bleeds before and after delivery have been reduced from 15 to 5% (52).

THE PRESENT POSITION

Production of sufficient quantities of anti-D IgG is no longer a problem in most countries that need it (53). It must be used in a dose high enough to prevent immunization, but its cost makes it important to find the lowest effective dose. The answer to this problem should be available in the near future when the results of the Medical Research Council's controlled trial of anti-D dosage are published. From other studies it would seem that a dose of 100 μg anti-D IgG given within 36 hr after delivery will reduce the Rh immunization rate by that pregnancy to about 1% instead of the 10% in untreated controls.

Much data indicate that many of these apparent failures of prophylaxis have been due to the onset of Rh immunization during the pregnancy. Thus, the anti-D had been given too late to have any chance of preventing immunization. Prophylactic anti-D given during pregnancy has been shown not to harm an Rh-positive fetus and trials are under way to test if injections at the 28th and 34th week of pregnancy reduce this remaining 1% immunization. Even if intrapregnancy immunization can be successfully eliminated, there are two other sources of Rh immunization that remain to be prevented. The first is blood transfusion, although large doses of anti-D IgG have been found to be effective in cases of accidental transfusion with Rh-positive blood (54). The other, and much more important, source is abortion.

As the incidence of immunization, and thus of Rh hemolytic disease, resulting from stimulation of full-term pregnancies falls because of Rh prophylaxis, and as the frequency of therapeutic abortions increases, we might expect to see a very important change in the natural history of Rh hemolytic disease. There is a real risk of an increased frequency of antibodies in the first full-term pregnancy. It has thus become a widely adopted policy to treat all Rh-negative women without detectable antibody with anti-D IgG following a therapeutic abortion. The dose used varies from 50–300 μg. It is likely that transplacental hemorrhage of a degree not protected against by about 100 μg anti-D must be very rare in this situation. Although spontaneous abortion is probably associated with a considerably lower risk than induced abortion, one must accept that prophylactic therapy always involves unnecessary treatment for many individuals. Rh-negative women should, therefore, be treated after any type of abortion unless the father is known to be Rh-negative. There will be general agreement that no risk should be taken with young nulliparous women in whom Rh immunization could result in their never having a live child.

ACKNOWLEDGMENTS

We wish to thank Dr. J. M. Bowman and Dr. M. G. Davey for allowing us to quote from current work in progress. We are grateful to Blackwell Scientific Publications Ltd. for permission to reproduce Figure 1.

Literature Cited

1. McConnell, R. B. 1966. *Ann. Rev. Med.* 17:291–306
2. Combined study from centers in England and Baltimore. 1966. *Brit. Med. J.* 2: 907–14
3. Kleihauer, E., Braum, H., Betke, K. 1957. *Klin. Wochenschr.* 35:637–38
4. Woodrow, J. C. 1970. *Ser. Haematol.* 3: 9–15
5. Bartsch, F. K. 1972. *Acta Obstet. Gynecol. Scand. Suppl.* 20:1–128
6. Woodrow, J. C., Finn, R. 1966. *Brit. J. Haematol.* 12:297–309
7. Merritt, K., Galton, M. 1969. *Transplantation* 7:562–66
8. Woodrow, J. C., Elson, C. J., Donohoe, W. T. A. 1971. *Nature* 233:62–63
9. Schneider, J. 1972. *Z. Geburtsh. Perinatol.* 176:2–16
10. Eklund, J., Nevanlinna, H. R. 1973. *J. Med. Genet.* 10:1–7
11. Hindemann, P. 1966. *Geburtsh. Frauenheilk.* 26:1359–72
12. Bishop, G. J., Krieger, V. I. 1970. *Med. J. Aust.* 1:663–68
13. Woodrow, J. C. See Ref. 4, 22–26
14. Woodrow, J. C. See Ref. 4, 17–18
15. Woodrow, J. C. See Ref, 4, 115–17
16. Cruchaud, A., Unanue, E. R. 1971. *J. Immunol.* 107:1329–40
17. Unanue, E. R., Cerottini, J. C. 1970. *J. Exp. Med.* 131:711–50
18. Argyris, B. F. 1969. *J. Reticuloendothel. Soc.* 7:500–17
19. Ryder, R. J. W., Schwartz, R. S. 1969. *J. Immunol.* 103:970–78
20. Sohnle, P. G., Sussdorf, D. H. 1972. *Immunology* 23:361–74
21. Pierce, C. W. 1969. *J. Exp. Med.* 130: 365–79
22. Möller, G. 1963. *J. Nat. Cancer Inst.* 30:1153–75
23. Pollack, W., Gorman, J. G., Hager, H. J., Freda, V. J., Tripodi, D. 1968. *Transfusion* 8:134–45
24. Brody, N. I., Walker, J. G., Siskind, G. W. 1967. *J. Exp. Med.* 126:81–91
25. Stern, K. 1969. *Clin. Exp. Immunol.* 4: 253–64
26. Henney, C. S., Ishizaka, K. 1968. *J. Immunol.* 101:896–904
27. Kappler, J. W., Hoffmann, M., Dutton, R. W. 1971. *J. Exp. Med.* 134:577–87
28. Walker, J. G., Siskind, G. W. 1968. *Immunology* 14:21–28
29. Siskind, G. W., Dunn, P., Walker, J. G. 1968. *J. Exp. Med.* 127:55–66
30. Mollison, P. L., Hughes-Jones, N. C. 1967. *Immunology* 12:63–73
31. Chang, H., Schneck, S., Brody, N. I., Deutsch, A., Siskind, G. W. 1969. *J. Immunol.* 102:37–41
32. Cerottini, J. C., McConahey, P., Dixon, F. J. 1969. *J. Immunol.* 102:1008–15
33. Sinclair, N. R. St. C. 1969. *J. Exp. Med.* 129:1183–1202
34. Rowley, D. A., Fitch, F. W. 1964. *J. Exp. Med.* 120:987–1005
35. Feldmann, M., Diener, E. 1970. *J. Exp. Med.* 131:247–74
36. Clarke, C. A. et al 1963. *Brit. Med. J.* 1:979–84
37. Preisler, O., Schneider, J. 1964. *Geburtsh. Frauenheilk.* 24:124–31
38. Gorman, J. G. 1971. *Clin. Obstet. Gynecol.* 14:635–46
39. Combined study from centers in England and Baltimore. 1971. *Brit. Med. J.* 2:607–9
40. Freda, V. J. 1971. *Clin. Obstet. Gynecol.* 14:594–610
41. Schneider, J. 1971. *Geburtsch. Frauenheilk.* 31:493–522
42. de Wit, C. D., Borst-Eilers, E., Van der Weerdt, C. M., Kloosterman, G. J. 1968. *Brit. Med. J.* 4:477–79
43. Woodrow, J. C., Clarke, C. A., McConnell, R. B., Towers, S. H., Donohoe, W. T. A. 1971. *Brit. Med. J.* 2:610–12
44. Maycock, W. d'A. 1970. Quoted in Ref. 53
45. Davey, M. G. 1973. Personal communication
46. Mollison, P. L. 1972. *Brit. Med. J.* 3: 31–34
47. Jones, A. R., Silver, S. 1958. *Blood* 13: 763–72
48. Polesky, H. F., Sebring, E. S. 1971. *Transfusion* 11:162–65
49. Cohen, F., Zuelzer, W. W. 1964. *Vox Sang.* 9:75–78

50. Bowman, J. M. et al 1970. *Pediat. Res.* 3:375–76
51. Zipursky, A., Israels, L. R. 1967. *Can. Med. Assoc. J.* 97:1245–57
52. Bowman, J. M. 1970. *Brit. J. Haematol.* 19:653–55
53. Clarke, C. A., McConnell, R. B. 1972. *Prevention of Rh-Hemolytic Disease.* Springfield: Thomas
54. Bowman, H. S., Mohn, J. F., Lambert, R. M. 1972. *Vox Sang.* 22:385–96
55. Woodrow, J. C., Donohoe, W. T. A. 1968. *Brit. Med. J.* 4:139–44
56. Borst-Eilers, E. Quoted by Mollison, P. L. in Ref. 46
57. Poulain, M., Huchet, J. 1971. *Rev. Fr. Transfus.* 14:219–24
58. Freda, V. J., Gorman, J. G., Galen, R. S., Treacy, N. 1970. *Lancet* 2:147–48
59. Queenan, J. T., Gadow, E. C., Lopes, A. C. 1971. *Am. J. Obstet. Gynecol.* 110: 128–30
60. Visscher, R. D., Visscher, H. C. 1972. *Am. J. Obstet. Gynecol.* 113:158–65
61. Queenan, J. T., Shah, S., Kubarych, S. F., Holland, B. 1971. *Lancet* 1:815–17
62. Matthews, C. D., Matthews, A. E. B., Gilbey, B. E. 1969. *Lancet* 2:318–19
63. Jorgensen, J. 1969. *Lancet* 2:1253–54
64. Wallace, J., MacDougall, J. Y. 1970. Personal communication
65. Murray, S., Barron, S. L. 1971. *Brit. Med. J.* 3:90–92
66. Ascari, W. Q. 1971. *Clin. Obstet. Gynecol.* 14:625–34
67. Gavin, P. S. 1972. *Obstet. Gynecol.* 39: 37–40
68. Freda, V. J. 1971. *Clin. Obstet. Gynecol.* 14:594–610
69. Mollison, P. L. 1972. *Blood Transfusion in Clinical Medicine.* Oxford: Blackwell. 4th ed.

Copyright 1974. All rights reserved

MARROW TRANSPLANTATION FOR APLASTIC ANEMIA AND ACUTE LEUKEMIA[1]

❖7106

P. Neiman, M.D., E. D. Thomas, M.D.,[2] C. D. Buckner, M.D., R. Storb, M.D., A. Fefer, M.D.,[3] H. Glucksberg, M.D., and K. G. Lerner, M.D.

Division of Oncology, Department of Medicine, University of Washington School of Medicine and United States Public Health Service Hospital, Seattle, Washington

INTRODUCTION

The effect of transplantation of marrow has been a subject of fruitful study for two decades. Such work in animal systems has led to observations of fundamental importance to the understanding of the hematopoietic and immune systems. This basic information, which has been recently reviewed (van Bekkum & de Vries 1, Trentin 2), underlies the principal subject of this article, namely the clinical use of engrafted marrow cells as an experimental approach to the definitive treatment of primary lethal disease of the marrow organ. Our principal focus will be on marrow aplasia and acute leukemia because most of our recent experience lies with these problems, although deficiency states of the immune system have also been approached by the technique of marrow transplantation in recent years (Buckley 3). These transplants performed on children with the rare combined immunodeficiency syndromes are of considerable scientific interest since some of the immunologic barriers to successful transplantation are compromised in such individuals. The application of marrow transplantation to aplastic anemia and acute leukemia considerably enlarges the number of patients who might benefit from this procedure.

---

[1] This investigation was supported by Public Health Service Research Grants CA 10895 and CA 10167 from the National Cancer Institute, Contract PH 43-67-1435 from the National Institute of Allergy and Infectious Diseases, and Contract NOI-CP-3-3236 within the Virus Cancer Program of the National Cancer Institute.

[2] Dr. Thomas is the recipient of Research Career Award AI 02425 from the National Institute of Allergy and Infectious Diseases.

[3] Dr. Fefer is a scholar of the Leukemia Society of America.

The technical challenge, however, is also enlarged since such immunologically mature individuals present the full spectrum of problems confronting the field of marrow transplantation. We shall attempt to review these difficulties here.

Following the initial observation of the rescue of lethally irradiated mice from death from marrow failure by engraftment of transplanted hemopoietic cells (Jacobson, Marks & Gaston 4, Lorenz et al 5), several attempts were made to achieve hemopoietic grafts in human beings beginning in the late 1950s (Thomas et al 5, Mathé et al 7). Review of this experience (Bortin 8) indicates that most of these attempts failed because of a series of obstacles including marrow graft rejection, graft-versus-host disease, and the consequences of marrow failure—infection and hemorrhage. With the development of practical new concepts and techniques in the areas of histocompatibility typing, immunosuppression, blood component therapy, and antibiotics, coupled with nearly fifteen years of experience with "clinical" marrow transplantation in outbred animal species, a second phase of human marrow transplantation has been initiated. Over the past four years our group has undertaken over 90 marrow transplants in patients with acute leukemia and primary marrow failure. While this effort is still in a phase of rapid evolution, and a summary of current results may not fully reflect even the immediate future, a review of the present state of affairs demonstrates considerable progress and provides a basis for some optimism.

ISOGENEIC MARROW TRANSPLANTATION

Marrow Aplasia in Twins

The least complex situation to be discussed is that of a patient who develops severe marrow failure and who has available to him isogeneic marrow from an identical twin to replace his own. Presently, 10 cases have been described involving patients with acquired aplastic anemia (Pillow et al 9), plus one individual who was accidentally exposed to doses of irradiation sufficient to produce fatal marrow damage (Thomas et al 10), where marrow from an identical twin was transplanted. In two instances, the patients died within a few hours or days. In 7 of the remaining 10 cases there was prompt recovery of hemopoietic function and apparent normal survival. Although proof of engraftment cannot be obtained in the isogeneic situation, the circumstantial evidence strongly supports the contention that engraftment of the infused marrow cells prevented death from marrow failure. In the rare circumstance that such conditions occur in an identical twin, marrow transplantation seems indicated as a life-saving procedure.

Acute Leukemia in Twins

The use of marrow transplants between identical twins to treat hemopoietic malignancy raises the problem of destroying the neoplastic marrow population in addition to that of reconstitution of normal hemopoiesis. Our experience with this problem has been recently reviewed in detail (Fefer et al 11). Three attempts have been made to treat subjects with acute lymphoblastic leukemia with supralethal doses of whole body irradiation followed by successful engraftment of isogeneic marrow from an identical twin. Unfortunately leukemia recurred in all three instances in 48 to 84 days. Subsequently, several modifications were introduced to improve these results based on the assumption that the mechanism of failure was insufficient tumor cell destruction. First, additional cytotoxic treatment has been given in the form of chemotherapy prior to whole body irradiation. Second, an attempt at attacking a residual leukemic cell population through immunotherapy following transplantation has been undertaken. This latter technique is based on principles derived from successful chemo-immunotherapy of murine leukemia in an experimental model system (Fefer 12, Fass & Fefer 13), and involves "immunization" of the marrow graft recipient with killed autologous leukemic cells coupled with infusions of additional lymphocytes from the marrow donor for several weeks following engraftment. No improvement in results was obtained in five patients where either chemotherapy or immunotherapy was added to whole body irradiation. Perhaps surprisingly, the combination of cyclophosphamide prior to irradiation and transplantation followed by immunotherapy in the post-transplantation period has produced some interesting results. Of ten patients treated, five have relapsed in periods of 27 to 204 days. The other five remain in remission for periods of six months to three years. Admittedly the number of patients are, necessarily, very small, and the mixture of different types of acute leukemia adds to the difficulty in drawing any but the most tentative conclusions about the relative efficacy of any component of the therapeutic regimen. Nevertheless, considering that all of these patients had exhausted conventional modalities of treatment, and considering the relative ease of the treatment procedure, marrow transplantation seemed to confer significant therapeutic benefit in a substantial fraction of these individuals. Controlled studies now in progress should provide information of general significance regarding the relative importance of cytotoxic and putative tumor-specific immunotherapy in this unique setting.

Reinfusion of Autologous Marrow

Closely related to the problem of identical twin transplants is the idea of autologous marrow reinfusion in support of marrow toxic anticancer treatment. This concept is based upon a number of precepts. First, animal marrow can be successfully frozen in medium containing glycerol or dimethyl sulfoxide, stored for varying periods of time up to several years, and used, subsequently, to reconsitute marrow and immunologic function in lethally irradiated recipients (Storb & Thomas 14). Human marrow stored in similar fashion can be shown to synthesize hemoglobin in vitro in response to stimulation by erythropoietin (Adamson & Storb 15), Second, a number of solid tumors, including neuroblastomas, solid lymphomas, some testicular tumors, and oat cell carcinoma of the lung are often quite sensitive to irradiation, and to some chemotherapeutic agents, limited principally by marrow tolerance. Therefore, in theory stored autologous marrow from patients with these diseases who lacked marrow involvement could be used to reconstitute marrow and immunologic function following otherwise lethal doses of irradiation and/or drug treatment. Only a very limited relevant clinical experience is available to date in man. One study, utilizing high-dose cyclophosphamide (120 mg/kg) to treat disseminated ovarian cancer failed to show benefit from marrow reinfusion because of a surprisingly rapid recovery of marrow function in control patients at this dose level (Buckner et al 16). Another ongoing study involves storage of marrow from patients in the benign chronic phase of chronic myelogenous leukemia. Such marrow has been reinfused in two of the original donors, following whole body irradiation, in an attempt to treat their terminal blastic transformation. In one instance there was prompt reconstitution of marrow function. When the patient died of a viral interstitial pneumonia three months later, there was no evidence of recurrent blastic crisis. The second patient had acute myelofibrosis accompanying his blastic transformation and expired after the irradiation without recovering marrow function. Similar attempts are planned for the future for patients with blastic transformation of chronic myelogenous leukemia who do not have extensive myelofibrosis.

ALLOGENEIC MARROW TRANSPLANTATION

General Principles

The problem of transplanting organs between genetically different individuals introduces the thorny problem of histoincompatibility. With respect to engraftment of immunocompetent tissue such as marrow, such incompatibility can be expressed not only as graft rejection, but also as an immunologically mediated reaction by the graft against host tissues, called graft-versus-host (GVH) disease. Progress in two areas has permitted successful allogeneic marrow grafts, at least between siblings. The first is the availability of practical techniques for the identification of genetic loci which specify at least one of the major transplantation antigen systems that mediate graft rejection and GVH reaction. This involves microcytotoxicity tests using specific antisera for the HL-A antigens on the surface of lymphocytes (Terasaki 17), and lymphocyte reactivity in mixed leukocyte culture (Bach & Amos 18), which appears to detect another closely linked major transplantation antigen system (Amos & Bach 19). Our policy has been initially to restrict allogeneic transplantation to siblings matched for HL-A antigens and unreactive in mixed leukocyte cultures. Unfortunately, graft rejection and GVH reaction can be mediated by other antigen systems that are not detected by these techniques, although extensive studies in the canine model system indicate that GVH reactions are less frequent, and less severe when they do occur, when donor-recipient pairs are matched by similar criteria (Storb et al 20). Therefore, a major degree of immunosuppression is required to prepare even matched recipients for marrow transplantation, and further post-grafting immunosuppression is required to prevent and ameliorate GVH reactions. Again, the treatment programs adopted for human use rely heavily on analogous regimens that are successful in animal models. Thus, the clinical use of whole body irradiation for marrow graft preparation, and methotrexate for post-transplantation suppressive of GVH reactions, is based on extensive experience in canines (Lochte et al 21, Thomas et al 22), while the use of cyclophosphamide for both purposes is derived from murine experiments (Santos et al 23).

Aplastic Anemia

The extension of marrow transplantation from the isogeneic setting to matched siblings considerably increases the potential number of patients who might benefit from this form of treatment. The genetics of the major histocompatibility loci are such that an otherwise suitable patient has a 25% chance of being matched with each available sibling. Thus, over the past three years we have been able to perform a series of 26 allogeneic marrow transplants between histocompatible siblings for the treatment of marrow aplasia. This experience has recently been described in detail (Storb et al 24). Briefly, 19 of the patients were prepared with cyclophosphamide and the rest with whole body irradiation. Twelve of the patients have avoided the complications of marrow graft rejection, opportunistic infection, and lethal GVH disease of the immediate post-grafting period, and were discharged to resume a reasonably normal existence. One patient died suddenly of unknown causes after 14 months, and the rest have continued to survive with good marrow function for periods of 4 to 27 months. Four of the patients had evidence of hepatitis before developing marrow failure, three had been exposed to suspect chemicals, and the rest had idiopathic marrow aplasia with the exception of one with paroxysmal nocturnal hemoglobinuria who is among the long-term survivors. Since in most instances long-term engraftment could be documented with cytogenetic and/or red cell antigen markers, these data indicate that aplastic anemia is, in most instance, a primary stem cell defect rather than an abnormality of the marrow microenvironment, or secondary to a persistent toxin or to the lack of an essential nutrient.

A number of issues are raised by this experience, not the least of which is a consideration of the evaluation of the role of marrow transplantation from that of a radical experiment to some permanent place in the definitive management of marrow aplasia. The precise definition of that place is dependent upon a clear understanding of a natural history of the disease, or group of diseases, called aplastic anemia. While a discussion of the state of knowledge in this respect is beyond the scope of this review, it is fair to say that no such clear understanding now exists. Nevertheless, an informal review of available statistics based on any reasonable definition of *severe* aplastic anemia in adults or children indicates a mortality rate from the consequences of marrow failure or leukemic transformation in excess of 90%. Our initial policy with regard to selecting candidates was to restrict this procedure to terminal and pre-terminal patients, accounting for the fact that 11 of the patients in this series were felt to present poor or very poor prospects for success on grounds of general condition and other factors. The emergence of the present experience has led us to propose and carry out this procedure for patients in better condition whose marrow function has declined to the point of requiring red cell or platelet transfusions (or to granulocyte levels below 500/mm^3) on an indefinite basis, and who have failed to respond to an adequate trial of androgens and other hematinics. The acquisition of further information on prognosis as it correlates with various manifestations of aplastic anemia may ultimately demonstrate that these standards are still too conservative, especially with respect to androgen therapy. Finally, it is not unreasonable to expect further improvement in the safety of marrow transplantation based on improved management of the lethal complications of this procedure, as will be discussed in subsequent sections.

Hematologic Malignancy, General Considerations

The use of immunocompetent allogeneic marrow transplants following massive cytotoxic antileukemic therapy introduces the possibility of an immunologic antileukemic effect of marrow transplantation over and above the effect of rescue from lethal marrow suppression. Such adoptive immunotherapy might be directed at putative tumor-specific antigens in leukemic cells [evidence for which is now accumulating (Powles et al 25, Fridman & Kourilsky 26, Rosenberg et al 27)] and/or result nonspecifically from the GVH reaction. Both mechanisms have been demonstrated to produce complete destruction of small populations of leukemic cells in murine model systems (van Bekkum & de Vries 1, Buckley 3, Rosenberg et al 27, Boranic 28, Bortin, Rimm & Saltzstein 29). This rationale is the basis for the suggestion that allogeneic marrow transplants might yield results in leukemias superior to those attempts achieved with transplants between identical twins. Although a reasonable number of such transplants have been carried out, the answer to the experimental proposition is, for a variety of reasons, still not apparent. The situation is, however, sufficiently promising to warrant a brief summary.

Leukemic patients have been prepared for transplantation by several different methods including whole body irradiation, massive combination chemotherapy (Graw et al 30), and high-dose cyclophosphamide as a single agent (Santos et al 31). Regarding the last therapy, cytogenetic analyses in the canine model system indicated that both host and donor hemopoietic and lymphoid cells could be detected following cyclophosphamide-prepared marrow transplants (Storb et al 32), while only donor-type cells have been detected in long-term follow-up of successful marrow transplants in canines prepared by irradiation (Storb & Thomas 14). Although the incidence, if any, of this cyclophosphamide-induced "mixed chimerism" in human marrow transplants remains to be determined (Santos et al 33), such persistence of host hemopoietic tissue seems undesirable. Therefore, our group has based its initial efforts to treat acute leukemia by marrow transplantation on whole body irradiation. Patient selection has generally been on the basis of refractoriness to conventional chemotherapy. A small number of cases were undertaken in remission, where the patient had suffered multiple previous relapses, and the duration of remission was expected to be short. Thus, 25 of the 46 leukemic patients discussed in the following sections were judged to be poor candidates for this procedure on grounds of general condition, and other factors which were thought to limit the prospects for success. All of these patients received marrow from siblings who were histocompatible by the criteria of serotyping for HL-A antigens and nonreactivity in mixed leukocyte culture.

Acute Lymphoblastic Leukemia

Our group has performed allogeneic marrow transplants on 24 patients with acute lymphoblastic leukemia, the first 6 of whom form a homogeneous group for subsequent comparison. These were children, 3 in relapse and 3 in remission, who were prepared for transplantation with 1000-rad whole body irradiation alone. All 6 achieved functional transplants, were initially free of leukemia, and had an uncomplicated post-transplantation course with the exception of mild GVH reactions in 2. One patient remains alive and free of disease for three years at the time of this writing. The rest developed recurrent disease in periods of 85 to 168 days post-grafting and have died. In two female patients, we have been able to establish by cytogenetic study that the recurrent leukemia was composed of male cells—the same sex as the donor (Fialkow et al 34, Thomas et al 35). In the other three instances the absence of markers and other problems have precluded establishing the origin of the recurrent leukemia. The potential mechanisms and significance of these findings have been discussed, but, needless to say, such a finding in 2 of 6 comparable cases requires some reassessment of our approach. Favoring, for the moment, the suggestion that such an event represents transmission of an etiologic virus, or subviral infectious material, from the leukemic patient to the engrafted marrow cells, hoping that the principal reservoir for such an agent was the original leukemic cell population, and noting that irradiation is an effective inducer of oncogenic viruses in animal model systems (Kaplan 36), we have modified our protocol for preparation for engraftment to include 60 mg/kg cyclophosphamide on each of two successive days beginning five days before the irradiation. This appears to be reasonably well tolerated and might be expected to improve results based on the following rationale. First, alkylating agents have been found in some systems to be poor inducers of occult potentially oncogenic viruses (Rowe et al 37). Thus, reducing the leukemic cell population by this means, before irradiation, might decrease the risk of release of a sufficient quantity of infectious material following irradiation to cause marrow graft transformation. Second, the additional cytoreduction achieved with cyclophosphamide could increase the chances of bringing the original leukemic cell population down to a level where the putative immunotherapeutic properties of the marrow graft might be operative and prevent host-type leukemic relapses. We have carried out this combined procedure on an additional ten children and seven adults with acute lymphoblastic leukemia at the time of this writing. Nine have expired from the complications of marrow transplantation in periods of 10 to 107 days without evidence of leukemia. Five patients are alive with normal marrow examinations for periods of 2 to 17 months post-grafting. Three patients have developed recurrent leukemia. Two of these recurred within a few weeks of grafting; serial marrow examination failed to demonstrate clearing of leukemic cells and, in one, available cytogenetic markers proved the recurrent disease was of host origin. We have interpreted these two patients as showing a leukemic cell population hopelessly refractory to the cytotoxic therapy. The third relapse occurred 95 days after transplantation, but there were, unfortunately, no markers available to establish the mechanism of the relapse. Thus, the problem of the frequency and significance of marrow graft transformation following this new regimen remains undetermined. Further, the demonstration of leukemic cell populations that are refractory to high-dose cyclophosphamide plus 1000-rad whole body irradiation predicts a wide range in the magnitude of cytoreduction achieved with this regimen, particularly in these end-stage patients. It seems likely, therefore, that regrowth of the original leukemic cell population will occur in some fraction of the transplantation survivors, even if the assumptions with respect to the adoptive immunotherapy of marrow transplantation are correct, simply because of inadequate reduction of the leukemic cell population. The size of the fraction of long-term leukemia-free survivors, if any, remains to be determined.

Attempts have been made to treat recurrent leukemia. In the two instances of documented marrow graft transformation, chemotherapy with previously ineffective agents was initiated with virtual disappearance of the leukemic cell population. Unfortunately the residual engrafted normal marrow proved very sensitive, and the patients expired from the consequences of therapy-induced marrow failure. In four recurrences where markers were not available, repeat marrow transplants were attempted following preparation with a cytotoxic multiple-drug combination based on that described by Graw et al (30). One patient survived the procedure, and achieved a successful transplant, but she developed recurrent disease and died five months later. Another is presently in the first few weeks post-transplantation, and the other two expired from sepsis within the first few days.

Acute Myeloblastic Leukemia

We have approached acute myeloblastic leukemia with the same rationale and technique used for acute lymphoblastic leukemia. At this time some 22 acute myeloblastic leukemia patients have received allogeneic marrow transplants from histocompatible siblings. The first four were prepared with whole body irradiation alone, but all of these succumbed to the complications of pancytopenia and GVH disease so that there is no experience with this single modality comparable to that observed in childhood acute lymphoblastic leukemia. The rest of the patients were prepared with the combination of cyclophosphamide and irradiation. Of these, ten died with the complications of allogeneic marrow transplantation in periods of 15 to 106 days post-grafting with no evidence of recurrent leukemia. Six patients are alive with good marrow function and free of leukemia for periods of 2 to 15 months. One patient demonstrated the refractory leukemia phenomenon and never achieved a marrow free of myeloblasts. Recurrence was diagnosed in one patient 95 days after transplantation, but, again, there are no markers available to establish the origin of the leukemic cells. Thus, we have not detected marrow graft transformation yet in this type of leukemia. The considerations with respect to the long-term prognosis for marrow graft survivors in this group of leukemic patients are similar to those discussed for the patients with acute lymphoblastic leukemia. Nevertheless, some complete and persistent remissions have been achieved in both groups of refractory end-stage patients with acute leukemia, giving some hope that continued progress in marrow transplantation will improve the prospects for these individuals, and improve the general prognosis for this group of lethal diseases.

Other Hematologic Malignancies

The rationale for allogeneic marrow transplants in acute leukemia can be applied with equal justification to other malignant states such as lymphocytic and histiocytic lymphoma, Hodgkin's disease, and blastic transformation of the myeloproliferative disorders. However, only a small number of such patients have undergone marrow transplantation, and to date there are no long-term survivors.

MECHANICS OF MARROW TRANSPLANTATION

The Clinical Setting

Even a superficial review of the experience described in the sections above demonstrates that, despite an advance over results obtained in the 1950s, long-term survival among recipients of allogeneic marrow transplants remains unsatisfactorily low. The subsequent sections deal with the methodology that has led to the present level of success, and the many obstacles that remain. The nature of the clinical setting in which successful marrow transplants have been achieved is, we believe, of central importance. Thus, most of the successful procedures have been performed in an intensive care center manned by a fully trained and experienced team of physicians, nurses, and technicians specifically committed to the project and fully equipped to provide the special supportive care techniques that are required. The complexity and uniqueness of some of the complications of marrow transplantation dictate that this procedure will continue to require such a special center to be successful in many of the patients. Nevertheless, the apparent clinical utility of allogeneic transplantation for marrow aplasia, for example, and the lack of available center beds for all patients who could conceivably benefit from the procedure dictates that marrow transplantation be carried out for some selected patients in the general medical setting of a university hospital, or in a comparably equipped and staffed medical center, in consultation with the transplantation center. A few such selected isogeneic and allogeneic marrow transplantation procedures have been carried out in our area outside of this special center with satisfactory results. While this experience is promising we emphasize, and will attempt to make even more clearly apparent, that marrow transplantation is still far too dangerous and complex to be undertaken in most "conventional" clinical settings.

Preparation, Pre- and Post-Grafting Immunosuppression

Whole body irradiation is carried out in our unit by opposing ^{60}Co sources to provide as uniform a dosimetry as possible, at a rate of 5 R/min to a midline dose of 1000 rad. The dosimetry is important both in terms of providing adequate immunosuppression to achieve engraftment (Thomas et al 22) and, in the case of leukemia, to achieve maximum reduction of the leukemic cell population. Although this dose is approximately twice the LD_{50} level for man, the acute extramedullary toxicity is usually quite mild. Most patients suffer only low grade fever, transient nausea and diarrhea and, usually, tender parotid swelling and xerostomia which resolve within the first 24 to 48 hr. A transient esophagitis is occasionally noted. The high-dose cyclophosphamide regimen, when used alone, consists of 50 mg/kg on each of four successive days beginning 24 hr after infusion of antigen from the prospective donor (usually in the form of a suspension of platelets and white blood cells). There is an interesting difference between cyclophosphamide (at this dosage level) and irradiation in this respect since exposure to donor antigen 24 hr before irradiation will block subsequent marrow engraftment (Storb et al 38). When 120 mg/kg cyclophosphamide is given to leukemic patients, three days are allowed to elapse before irradiation in the hope of avoiding cumulative toxicity to the intestinal mucosa or other organs. The nausea and vomiting induced with cyclophosphamide are usually quite severe, but transient. A high urine flow must be maintained, usually in excess of 250 cc/hr to avoid severe cystitis from urinary metabolites of this drug. An anti-diuretic effect of cyclophosphamide has been described (De Fronzo et al 39), and has been frequently seen in our experience, requiring the use of diuretics to maintain this high urine flow. Finally, care must be taken to avoid urate nephropathy in leukemic patients receiving this massive cytoreduction preparatory to engraftment.

Post-grafting immunosuppression is given to suppress GVH disease. Two regimens have been used, one based on cyclophosphamide (Santos et al 31), the other on methotrexate. We have exclusively used the latter in a planned dose schedule of 15 mg/m^2 intravenously on the day following marrow infusion and 10 mg/m^2 on days 3, 6, 11, and weekly thereafter to day 100 following transplantation. As mentioned, such a program has been demonstrated in controlled trials in canines to significantly reduce the incidence and severity of GVH disease (Lochte et al 21, Storb et al 40). With the possible exception of some immunodeficiency states, allogeneic transplantation in man should not be undertaken without post-grafting immunosuppression.

Technique of Marrow Aspiration and Reinfusion

The technique for obtaining marrow for transplantation has recently been reviewed (Thomas & Storb 41). Briefly, donors are given spinal, or occasionally general anesthesia, and 400 to 700 ml of marrow (in adults) is aspirated by multiple needle puncture from both anterior and posterior iliac crests using sterile surgical techniques. The blood volume is partially replaced with a unit of autologous whole blood previously obtained from the donor. The heparinized marrow suspension is passed successively through 0.3 and 0.2 mm mesh stainless steel screens to produce a suspension of single cells and small clumps. Infusion of unscreened marrow can produce fatal pulmonary embolization. From adult donors marrow yields vary from 10 to 30 billion nucleated cells. This suspension is infused intravenously without further filtration but with careful monitoring for evidence of pulmonary microemboli or right heart strain. Mild hypoxia and fever are not uncommon transient effects of marrow infusion, but no serious complications have occurred to date. The donor suffers only some soreness at the aspiration sites, and the small but definite risk associated with anesthesia.

Kinetics of Marrow Repopulation

Study of serial diagnostic marrow aspirates, biopsies, and autopsy specimens indicates that marrow repopulation is evident as early as seven days following infusion in the form of small focal accumulations of hemopoietic cells. Increase in marrow cellularity by growth and coalescence of these foci is slowly progressive over a period of several months thereafter. Fortunately production of adequate numbers of blood cells from the engrafted marrow occurs fairly rapidly after marrow infusion. The kinetics of recovery of production of white blood cells in 42 allogeneic marrow transplants are depicted in a computer-assisted analysis shown in Figure 1. The nadir of the white count occurs, on the average, between days 7 and 9 and the counts begin to climb thereafter such that mean granulocyte counts exceed 500/mm^2 by day 17. A wave form is present in this recovery curve with peaks just prior to methotrexate administration. Interestingly, there is no significant effect of total marrow dose (between the limits of 5 and 30 billion cells/m^2) administered to these patients. The recovery of adequate platelet and red blood cell production lags a little behind, such that platelet transfusions can be terminated on the average by day 25 (a SD of ± 11 days) and red blood cell transfusions by day 27 (a SD of ± 10 days). Although detailed studies of marrow function have not as yet been carried out after blood counts enter the normal range, there is ample anecdotal experience to suggest that the effective marrow reserve is limited for several months. This is expressed as the frequent development of cytopenia in response to any stimulus that increases the demand for blood cells such as infection, GVH disease, or the administration of cytotoxic antithymocyte serum. Thus, transient resumption of platelet or red blood cell transfusions is sometimes necessary during the first few months if the patient encounters these difficulties.

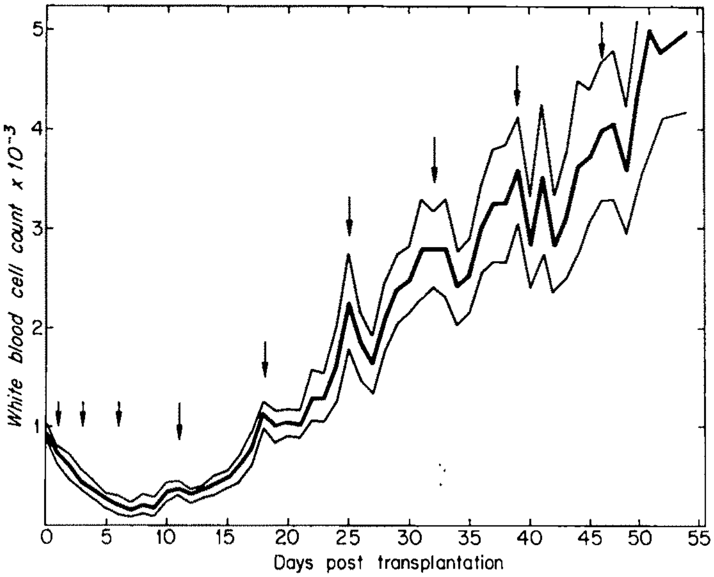


Figure 1 Kinetics of recovery of white blood cell counts following allogeneic marrow transplantation. The bold line represents mean values and the outer lines are the standard error determinations. The arrows represent the times of administration of methotrexate.

Reconstitution of Immunologic Function

Studies of the reconstitution of immunologic function following marrow transplantation in patients with marrow aplasia or leukemia are still incomplete, although some observations of several objective measurements of humoral and cellular immunity have recently been reported (Fass et al 42). These observations in a very small number of marrow transplant survivors indicate that these parameters of both cellular and humoral immunity are depressed but returned to normal by about nine months post-grafting in two of three transplants between identical twins. In apparent contrast, study of allogeneic transplant recipients indicated that some of these parameters, such as formation of humoral antibody to bacteriophage ΦX174 and delayed hypersensitivity to a battery of skin test antigens, including the potent haptene dinitrochlorobenzene, remain depressed in most cases beyond day 200. The status of immune function in these patients may be very complex, since antibodies against certain antigens (e.g. cytomegalovirus and allogeneic leukocytes) have been detected within the first few months after transplantation, sometimes in surprisingly high titer (Neiman et al 43). Further, reactivity of engrafted lymphocytes in mixed leukocyte culture is restored one to three months after grafting, and total immunoglobulin levels are generally restored by day 100. Nevertheless, opportunistic infections of the kind commonly encountered in immunosuppressed patients are very frequent complications in the group of allogeneic transplant recipients during the first four months after grafting. Striking lymphoid hypoplasia (despite normal numbers of circulating lymphocytes) in the lymph nodes and spleen is the rule at postmortem examination in patients who die of these infections. Even patients who are apparently well more than one year after grafting have had occasional difficulty with purulent infections such as otitis media, meningitis with *Hemophilus influenza,* and pneumococcal pneumonia; these have not thus far been fatal (Clift et al 44). The role of GVH disease and post-grafting immunosuppression in delaying immunologic reconstitution is suspected to be substantial, but considerable study remains to be done before the precise nature and causes of this important immunologic deficit will be fully defined. Techniques for accelerating the reconstitution of immune function (without exacerbating GVH reactions) based on these studies could be expected to decrease the mortality after marrow transplantation.

Supportive Management

Since the methods used to prepare marrow graft recipients eliminates hemopoiesis until the transplanted marrow begins to function at life-supporting levels, the need for transfusional support is obvious. Techniques for blood component therapy with red blood cells and platelets are well established, and routinely employed with certain special considerations. For example, such blood products contain immunocompetent lymphocytes that could produce deleterious GVH reactions following infusion into the immunosuppressed recipient. For that reason all allogeneic blood products are irradiated to 1500 rad to block this complication. Platelet transfusions often present problems because of immunization of these extensively transfused patients to platelets from random donors. In this setting the histocompatible marrow donor makes the ideal platelet donor following transplantation through the technique of multiple unit plateletpheresis. If this is not possible, any donor who does not have any HL-A antigens in excess of those found on the recipient cells may be used successfully (Graff & Yankee 45). The use of granulocyte transfusions is still experimental. Techniques are now available to obtain up to 1 to 3 billion granulocytes a day from normal donors through the use of on-line nylon wool packs (Djerassi et al 46) or specialized centrifuges (Clift et al 47). This usually involves placing a silastic arteriovenous shunt in the granulocyte donor. Thus, such donors are usually family members intimately involved in the patient's situation. Evidence is available that, in the absence of immunity, transfused granulocytes prepared by these techniques do circulate, are delivered to sites of inflammation, and do ingest and kill bacteria normally (Goldstein et al 48). Therefore, we have supported selected marrow graft recipients who had infection with granulocyte transfusions. Our anecdotal experience, coupled with other clinical reports (Graw et al 49) and some controlled experiments in animals (Epstein, Clift & Thomas 50), suggests that these transfusions are of benefit in managing bacterial infections in granulocytopenic recipients, although rigorous demonstration of the range of effectiveness of such granulocyte transfusions is still lacking.

One obvious technique for avoiding infections in susceptible patients is to isolate them from environmental pathogens. We have used the commonly practiced "mask and glove" reverse isolation techniques routinely for all transplant recipients, although there is little objective data that suggest that such limited procedures confer much protection. Recently, controlled studies with ultra-isolation techniques have suggested that laminar air flow isolation rooms combined with antibiotic intestinal sterilization regimens and sterile diets confer some protection from bacterial infection in leukemic patients receiving marrow toxic chemotherapy (Levine et al 51). We have preliminary evidence to the effect that marrow transplant recipients can tolerate such regimens, but as yet have no evidence that this procedure will reduce mortality from the spectrum of pathogens that infect these patients.

COMPLICATIONS OF MARROW TRANSPLANTATION

Effects of Irradiation and Cyclophosphamide

The acute toxicity of the various regimens used to prepare patients for marrow transplantation has been mentioned, and has usually not presented serious problems. To consider the long-term effects of irradiation and cyclophosphamide in a review such as this is some measure of the extent of progress in human marrow transplantation. The happy circumstance of the existence of a small number of long-term survivors of otherwise lethal marrow diseases does raise the issue of such long-term considerations as the effect of 1000-rad whole body irradiation on growth rate in children, development of cataracts, reproductive capacity, teratogenesis, or even carcinogenesis. Similar considerations can be raised for massive doses of alkylating agents with some additional considerations. For example, we have observed a single case of chronic hemorrhagic cystitis of late onset that has persisted for over four months in a marrow graft recipient prepared with the cyclophosphamide regimen. No more definitive statement can be made yet except to say that the experience thus far with the long-term survivors has been otherwise uneventful with respect to these considerations.

Infectious Complications

The consequences of infection present a major problem for the management of marrow graft recipients, and infection is the proximate cause of death in almost all of these patients. Our overall experience with infection has recently been reviewed (Clift et al 44). The problem begins at presentation in that about 40% of the cases we have described here have documented local or generalized infection with significant bacterial, fungal, or viral pathogens at the time of transplantation. The subsequent infectious complications can be characterized by these same categories. Bacterial (largely *Pseudomonas aeruginosa* and *Escherichia coli*) and fungal (largely *Candida*) sepsis presents a frequent problem during the period before day 30 when marrow graft function is inadequate. A second major association with blood stream infections following the establishment of the graft is the presence of debilitating GVH disease leading to extensive portals of entry through inflamed skin and damaged intestine. With antibiotics and the intensive supportive measures described in the previous sections, bacterial and fungal infections have only occasionally been fatal during the period before the marrow graft begins to function, unless the graft was rejected. Even in the setting of GVH disease, most of the mortality from sepsis occurs in the form of what appears to be an inevitable terminal event in a severely debilitated patient. Localized bacterial infections such as pneumonia, purulent sinusitis, perirectal abscess, and miscellaneous cellutitis have all been observed during the same period of time with the same general experience. Finally, the late incidence of suppurative infection in a few long-term survivors has been mentioned. All these episodes have been successfully treated. Thus, bacterial and fungal infections have contributed greatly to morbidity, but only occasionally to mortality except in the situations of marrow graft rejection or progressive debilitating GVH reaction.

Opportunistic infections of documented or presumed viral origin have presented an even more difficult problem. The most frequent manifestation, occurring in 40% of the marrow graft recipients, is interstitial pneumonia, which presents between day 1 and 116 post-grafting, and is associated with a 60% mortality from respiratory insufficiency. A detailed summary of our experience with this problem has recently been published (Neiman et al 43). Subsequent to that report we have become increasingly aware that enteritis of viral origin is another serious, if less frequent occurrence. Although some of these fatal episodes have terminated a period of GVH disease, others have proved fatal to patients who appeared to have resolved their GVH reaction, and to still other patients who were free of complications. In about one half of the cases, cytomegalovirus can be incriminated as the etiologic agent by culture and/or histologic examination of biopsy and autopsy material. There have been two episodes of fatal varicella-zoster pneumonia, and one case in which generalized infection with *Toxoplasma gondii* was noted along with cytomegalovirus. Our initial review of some 50 marrow graft recipients indicated a previous exposure to cytomegalovirus in 52%, and a minimum rate of infection or activation post-grafting of 31%. Although the source of the cytomegalovirus infection is not fully documented, massive and obligatory exposure to blood products used in support of these patients provides an adequate explanation for most of the cases. In this study we failed to detect any evidence of *Pneumocystis carinii* although in recent months several cases have been detected. This apparently low incidence of *P. carinii* is perhaps surprising although a recent review of the infectious complications in renal transplant recipients specifically linked this agent with the use of corticosteroids (Anderson et al 52), drugs which are used minimally, if at all, in marrow grafting. At present about half of our cases of interstitial pneumonia are unexplained. Recently, new endemic human viruses have been described which become clinically apparent in the immunosuppressed state (Gardner 53, Lecatas et al 54). Thus, the etiologic possibilities have not been exhausted. The underlying problem is, undoubtedly, the immunodeficiency of marrow transplant recipients. It seems reasonable to suggest, therefore, that reduction in mortality from this complication will await techniques for accelerating the reconstitution of immune function or, at least, techniques for more effective treatment of these opportunistic infections.

Marrow Graft Rejection

Studies in animal model systems and limited comparable experience in man demonstrate that properly immunosuppressed recipients rarely, if ever, fail to accept marrow transplants from donors who are histocompatible by criteria similar to those described in the previous section. Marrow graft rejection can be produced, however, by immunization of the prospective recipient to unshared "minor" transplantation antigens in the donor. With respect to the human situation, such sensitization might occur through exposure to multiple blood transfusions, one or more of which might come from an individual with such an unshared transplantation antigen. For example, multiple random blood transfusions have been observed to increase significantly the incidence of marrow graft rejection in the canine system (Storb et al 55). Prior exposure to blood from the prospective donor is catastrophic in this model, leading to rejection in the majority of animals (Storb et al 38). In humans, especially those with acute leukemia, blood transfusion therapy is often accompanied by immunosuppressive drug regimens. Thus, one is hard pressed to predict the effect of the usually extensive exposure to random alloantigens on marrow transplants in man. At the time of this writing, 6 of 45 evaluable allogeneic transplants in leukemic patients failed to function adequately. Aplastic anemia patients are less frequently subjected to intensely immunosuppressive drug regimens. Interestingly, 6 of 25 marrow graft recipients with aplastic anemia either showed no evidence of marrow graft function despite adequate (greater than three weeks) post-transplant survival, or evidenced complete disappearance of hemopoietic marrow cells a few days or weeks after achieving functional transplants. While a non-immune mechanism related to the aplastic disease process itself cannot be ruled out, this experience is roughly comparable to the observed effect of random blood transfusions administered prior to transplantation to normal canine recipients in the model system (Storb et al 55). Since avoidance of this rejection phenomenon would substantially increase survivorship, particularly in the aplastic anemia group, we have encouraged the referral of such patients for transplantation before they have been extensively transfused. Because of the uncertainty with regard to the natural history of marrow aplasia in any given patient, such a recommendation is sometimes difficult to follow. At a minimum, blood transfusions from family members should be strictly avoided (three of the six patients with aplastic anemia who rejected their transplants had previous exposure to family or marrow donor blood products). Very recently a new immunosuppressive regimen employing a combination of procarbazine, antithymocyte globulin, and irradiation has been demonstrated to overcome blood-transfusion-mediated presensitization in a substantial fraction of marrow graft recipients in the canine model system (Floersheim et al 56). Very preliminary experience with this regimen in man, involving marrow transplant recipients who had prior exposure to family or marrow donor blood cells, is encouraging. Much more testing will be required to determine if the fraction of long-term human survivors can be increased through this technique.

Graft-Versus-Host Disease

Our closing comments deal with the complication of marrow transplantation, alluded to in the opening paragraphs, which was anticipated to be the major obstacle to routine success of allogeneic marrow grafting—GVH reactions (Mathé 57). Experience with this problem indicates that GVH disease comes close to fulfilling this unhappy expectation, although there are a few encouraging new developments to point out. A survey of our cumulative experience over the past four years indicates that at this writing 41 of the 73 allogeneic transplant recipients with acute leukemia or aplastic anemia developed the GVH syndrome with histologic evidence on biopsy or autopsy confirming the clinical impression. If one discards the ten patients whose grafts failed to "take" or were rejected, then about two-thirds of human recipients of allogeneic marrow from donors matched at the genetic loci specifying HL-A antigens and reactivity in mixed leukocyte culture developed GVH disease. Clearly, there are additional genes that mediate this reaction, and which segregate independently from the alleles for the "major" determinants of histocompatibility. There is at present no method for detecting these genes. The clinical consequences of this incompatibility are, as expected, appreciable. The principal target organs are the skin, intestine, and liver The skin shows a generalized nonspecific erythematous rash which frequently involves the palms of the hands and soles of the feet. This can progress to bullous formation and generalized erythroderma. The intestinal manifestations include nausea, vomiting, malabsorption, voluminous diarrhea (varying from watery to bloody), abdominal pain, and ileus. Liver disease in these patients is complex, the most consistent findings being moderate hepatomegaly and direct hyperbilirubinemia. Indices of hepatocellular damage such as serum glutamic oxaloacetic transaminase (SGOT) frequently rise above normal levels at the same time that other signs of GVH reactions appear. Review of the cumulative data, however, indicates that the SGOT levels are often elevated to comparable levels in patients without documented GVH disease. A detailed review of the histologic picture of GVH disease in our patients has been presented

(Lerner et al 59). Briefly, the skin shows damage to the basal layer of the epidermis, and the intestine shows individual gland necrosis from pylorus to anus (with the most prominent lesions being in the small intestine). The most characteristic features in the liver are lesions involving the small bile ducts in the portal triads. Hepatocellular necrosis is variable, and all involved organs show a modest lymphoid infiltration, particularly around the damaged epithelial structures. The most advanced lesions show virtual destruction of the target organs. The clinical course of 18 allogeneic marrow transplant recipients with documented GVH disease is presented in Figure 2 in terms of clinical estimates of skin and intestinal damage, and serum bilirubin levels. These patients are an early fraction of the whole group of allogeneic marrow recipients who received methotrexate as the principal post-transplantation therapy (although in a few instances cyclophosphamide and/or corticosteroids were used, when the GVH reaction was far advanced, without apparent clinical benefit). One can appreciate from this computer-assisted analysis that the skin reactions tend to appear before the intestinal and hepatic lesions. The mortality during the first three months post-grafting in this group of patients was 100%, in comparison to 41% in a comparable group of 22 patients without GVH disease who were managed in our ward in identical fashion over the same time period.

Subsequent to this experience two new techniques have been introduced that appear to have modified this grim picture in a hopeful way. First, we have noted that parenteral hyperalimentation through silastic arteriovenous shunts or indwelling subclavian catheters can maintain the nutritional status of patients who otherwise rapidly waste and become debilitated due, in part, to the severe malabsorption that accompanies even modest degrees of intestinal GVH reactions. The second, and perhaps more fundamental modification is the use of antihuman thymocyte globulin prepared in rabbits or goats for the treatment of established GVH disease. As with most of the other useful techniques, this concept is derived from experiments in the canine model system where an analogous antiserum added to the methotrexate regimen was shown to depress GVH reactions and prolong survival in animals receiving marrow from deliberately mismatched donors (Storb et al 58). With this background we undertook to add antihuman thymocyte globulin to the methotrexate regimen whenever a marrow graft recipient developed what appeared to be a progressive GVH reaction. Progression to intestinal and liver involvement has been used as the indication for this therapy because a number of patients with mild transient skin rashes have survived without receiving additional immunosuppression. We have now treated some 20 patients in this manner, and a detailed analysis of the resulting experience is being prepared. Although the only control for this experiment is our previous experience, we share a strong impression that this technique has produced some modification of GVH reactions. Some patients have resolved their lesions rapidly and completely and joined the ranks of the long-term survivors. Others have had less rapid or complete improvement. The skin lesions appear to improve in virtually all instances while the intestinal and hepatic damage follows a more variable course following treatment. The overall effect of antihuman thymocyte globulin is reflected in an improved survivorship of 33% within this group for the first three months following transplantation. Unfortunately, the use of antihuman thymocyte globulin is not without some special complications of its own. Cytotoxic antibody in our preparations, particularly in the material prepared in goats, can produce thrombocytopenia and hemolytic anemia. Apparent depression of the marrow graft itself has been observed in both the animal model (Storb et al 58) and in man. In two instances the marrow became sufficiently hypocellular

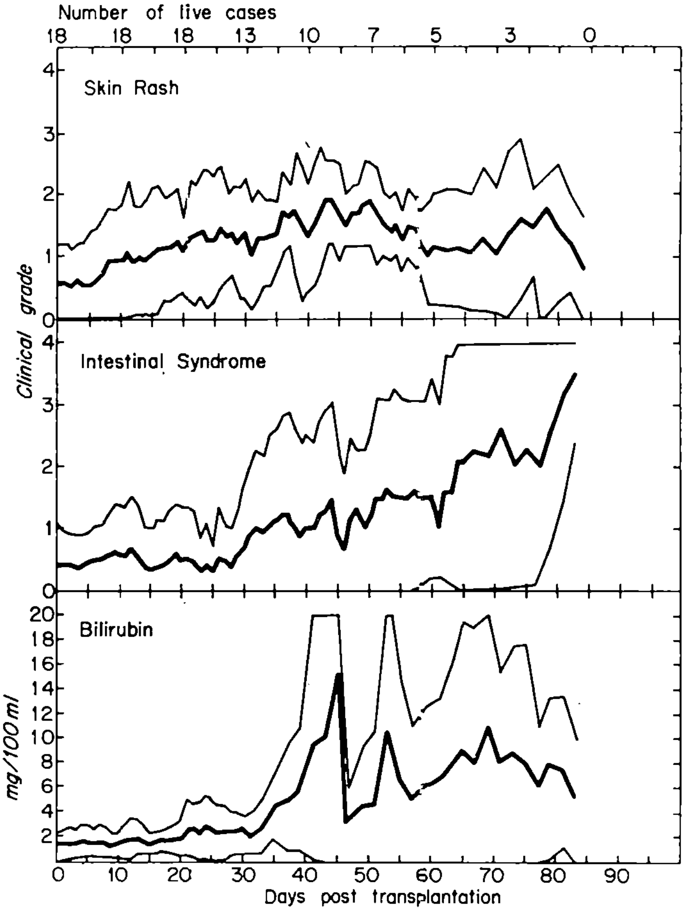


Figure 2 Graft-versus-host disease. Aggregate data on skin (upper panel), intestinal damage (middle panel), and serum bilirubin (lower panel) in 18 patients with histologic confirmation of GVH disease following allogeneic marrow transplantation. Clinical grading (0 = normal, 1 = mild, 2 = moderate, 3 = severe, 4 = life-threatening) was based on observations made by attending physicians at the time of occurrence. The middle bold line is the mean value and outer lines represent standard error determination.

that reinfusion of donor marrow was required. Only one of these patients survived this apparent toxic depression of marrow function. The profound immunosuppressive effects of antihuman thymocyte globulin on cellular immunity may add to the immunologic deficit already detected in marrow graft recipients, and thus render them even more susceptible to opportunistic infection. A precise measurement of the increase in infections, if any, following antihuman thymocyte globulin therapy is not available. However, we have observed a number of patients who appeared to be clearing their GVH lesions and then suffered a terminal setback in the form of interstitial pneumonia, exacerbation of hepatic damage, and increased evidence of enteritis. Autospy subsequently demonstrated the lesions of disseminated cytomegalovirus in several cases, infection which was the apparent proximate cause of death. Thus there is some progress in the management of GVH reactions, but there remains an enormous task ahead in better defining the pathophysiology of the GVH syndrome, in determining its relationship to immunologic deficiency and attendant opportunistic infection, and in improving the techniques for the management of this most vexing of complications.

Literature Cited

1. van Bekkum, D. W., de Vries, M. J. 1967. *Radiation Chimaeras.* New York: Academic
2. Trentin, J. J. 1972. *Exp. Hematol.* 22: 18–22
3. Buckley, R. H. 1971. *Progress in Immunology,* ed. D. B. Amos, 1061–80. New York: Academic
4. Jacobson, L. O., Marks, E. I., Gaston, E. O. 1951. *Quart. Rep. Div. Biol. Med. Res. Argonne Nat. Lab.* ANL-4625:46
5. Lorenz, E., Uphoff, D., Reid, T. R., Shelton, E. 1951. *J. Nat. Cancer Inst.* 12: 197–201
6. Thomas, E. D., Lochte, H. L. Jr., Lu, W. C., Ferrebee, J. W. 1957. *N. Engl. J. Med.* 257:491–96
7. Mathé, G. et al 1959. *Rev. Fr. Etud. Clin. Biol.* 4:675–704
8. Bortin, M. M. 1970. *Transplantation* 9: 571–87
9. Pillow, R. P., Epstein, R. B., Buckner, C. D., Giblett, E. R., Thomas, E. D. 1966. *N. Engl. J. Med.* 275:94–97
10. Thomas, E. D. et al 1971. *Exp. Hematol.* 21:16–18
11. Fefer, A. et al 1972. *Trans. Assoc. Am. Physicians.* In press
12. Fefer, A. 1973. In *Proceedings of the 26th Annual Symposium on Fundamental Cancer Research.* Houston: Univ. Texas. In press
13. Fass, L., Fefer, A. 1972. *Cancer Res.* 32: 997–1001
14. Storb, R., Thomas, E. D. 1972. *Proceedings of the Sixth Leukocyte Culture Conference,* ed. M. R. Schwarz, 805–40. New York: Academic.
15. Adamson, J. W., Storb, R. 1972. *Transplantation* 14:490–94
16. Buckner, C. D. et al 1972. *Cancer* 29: 357–65
17. Terasaki, P. I. 1970. *Histocompatibility Testing 1970.* Baltimore: Williams & Wilkins. 658 pp.
18. Bach, F. H., Amos, D. B. 1967. *Science* 156:1506–8
19. Amos, D. B., Bach, F. H. 1968. *J. Exp. Med.* 128:623–37
20. Storb, R. et al 1972. *Eur. J. Clin. Biol. Res.* 17:680–85
21. Lochte, H. L. Jr., Levy, A. S., Guenther, D. M., Thomas, E. D., Ferrebee, J. W. 1962. *Nature* 196:1110–11
22. Thomas, E. D., LeBlond, R., Graham, T., Storb, R. 1970. *Radiat. Res.* 41: 113–24
23. Santos, G. W., Burke, P. J., Sensenbrenner, L. L., Owens, A. H. Jr. 1969. *Excerpta Med. Found. Int. Congr. Ser.* 197:24–31
24. Storb, R. et al 1973. *Blood.* In press
25. Powles, R. L., Balchin, L. A., Fairley, G. H., Alexander, P. 1971. *Brit. Med. J.* 1: 486
26. Fridman, W. H., Kourilsky, F. M. 1969. *Nature* 224:277
27. Rosenberg, E. B. et al 1972. *Int. J. Cancer* 9:648–58
28. Boranic, M. 1968. *J. Nat. Cancer Inst.* 41:421–37

29. Bortin, M. M., Rimm, A. A., Saltzstein, E. C. 1973. *Science* 179:811–13
30. Graw, R. G. Jr. et al 1972. *Exp. Hematol.* 22:118–25
31. Santos, G. W. et al 1971. *Transplant. Proc.* 3:400–4
32. Storb, R., Epstein, R. B., Rudolph, R. H., Thomas, E. D. 1969. *Transplantation* 7:378–86
33. Santos, G. W. et al 1972. *Clinical Transplantation,* ed. D. H. Hume, 133–38. New York:Grune & Stratton
34. Failkow, P. J., Thomas, E. D., Bryant, J. I., Neiman, P. E. 1971. *Lancet* i:251–55
35. Thomas, E. D. et al 1972. *Lancet* i: 1310–13
36. Kaplan, H. S. 1967. *Cancer Res.* 27: 1325–40
37. Rowe, W. P., Lowy, D. R., Teich, N., Hartley, J. W. 1972. *Proc. Nat. Acad. Sci. USA* 69:1033–35
38. Storb, R., Epstein, R. B., Rudolph, R. H., Thomas, E. D. 1970. *J. Immunol.* 105:627–33
39. De Fronzo, R. A., Braine, H., Colvin, O. M., Davis, P. J. 1973. *Ann. Intern. Med.* 78:861–69
40. Storb, R., Epstein, R. B., Graham, T. C., Thomas, E. D. 1970. *Transplantation* 9: 240–46
41. Thomas, E. D., Storb, R. 1970. *Blood* 36:507–15
42. Fass, L. et al 1973. *Transplantation* 16:630–40
43. Neiman, P. et al 1973. *Transplantation* 15:478–85
44. Clift, R. A. et al 1973. *Transplant. Proc.* In press
45. Graff, K., Yankee, R. A. 1973. *Transplant. Proc.* In press
46. Djerassi, I., Kim, J. S., Mitrakul, C., Suvansri, U., Ciesielka, W. 1970. *J. Med.* 1:358–64
47. Clift, R. A., Buckner, C. D., Williams, B. M., Hickman, R. O., Thomas, E. D. 1973. *Transfusion* 13:276–82
48. Goldstein, I. M., Eyre, H. J., Terasaki, P. I., Henderson, E. S., Graw, R. G. Jr. 1971. *Transfusion* 11:19–24
49. Graw, R. G. Jr., Herzig, G., Perry, S., Henderson, E. S. 1972. *N. Engl. J. Med.* 287:367–71
50. Epstein, R. B., Clift, R. A., Thomas, E. D. 1969. *Blood* 34:782–90
51. Levine, A. S. et al 1973. *N. Engl. J. Med.* 288:478–83
52. Anderson, R. J., Schafer, L. A., Olin, D. B., Eickhoff, T. C. 1973. *Am. J. Med.* 54:453–60
53. Gardner, S. D. 1973. *Brit. Med. J.* 1: 77–78
54. Lecatas, G., Prozesky, O. W., van Wyk, J., Els, H. J. 1973. *Nature* 241: 343–44
55. Storb, R., Kolb, H. J., Graham, T. C., Kane, P. J., Thomas, E. D. 1972. *Transplantation* 14:248–52
56. Floersheim, G. et al 1973. *Exp. Hematol.* In press
57. Mathé, G. 1961. *Diagnosis and Treatment of Acute Radiation Injury,* 191–223. Geneva: World Health Organzation
58. Storb, R. et al 1973. *Blood* 62:601–9
59. Lerner, K. G. et al 1973. *Transplant. Proc.* In press

Copyright 1974. All rights reserved

PHARMACOTHERAPY OF SCHIZOPHRENIA

❖7107

Gerald L. Klerman, M.D.
Department of Psychiatry, Harvard Medical School; Psychiatric Service, Massachusetts General Hospital; and Erich Lindemann Mental Health Center, Boston, Massachusetts

INTRODUCTION

The treatment of mental illnesses, especially schizophrenia, has been dramatically changed since the early 1950s when the phenothiazines, the first of the new psychopharmacologic agents (often mistakenly called "tranquilizers"), were introduced. In 1952 chlorpromazine, the prototypic phenothiazine, was demonstrated in France to produce dramatic effects on the distressing symptoms and abnormal behaviors of schizophrenia, effects qualitatively different from those produced by previous treatments. Since then, over two dozen other compounds from eight different chemical classes have been shown to possess therapeutic efficacy in schizophrenia, and pharmacotherapy is now established as a necessary, if not always sufficient, component of any comprehensive treatment program for schizophrenia.

While pharmacotherapy does not "cure" schizophrenia in the same sense that the antibiotics cure syphilis or other infectious diseases, the available drugs do control most symptomatic manifestations and behavioral deviances, facilitate the patient's tendency towards remission, and improve his capacity for social, occupational, and familial adjustment. Clinically, the treatment of schizophrenic symptoms such as anxiety, delusions, hallucinations, paranoid states, catatonia, social withdrawal, and autonomic nervous system dysfunctions has been changed markedly by pharmacotherapy. The widespread use of these relatively safe compounds has contributed to greatly reducing the large number of chronic patients residing in public mental hospitals, shortening the duration of hospitalization for acute episodes, and shifting the focus of treatment of schizophrenia from institutional care to community based ambulatory treatment programs (1, 2).

In addition to these therapeutic effects, the advent of effective pharmacotherapy for schizophrenia has also influenced research methodology and theory. Because of the initial skepticism about the efficacy of these new compounds, considerable effort was expended to improve drug evaluation in psychiatry. With support and guidance from the NIMH Psychopharmacology Service Center (now the Psychopharmacology Research Branch), large scale programs were undertaken by the NIMH, the VA, and other systems, which contributed to what Hollister has called a "massive research overkill" (3, 4). Whatever may have been the reasons within the profession and in the larger mental health field requiring this "overkill," it had highly beneficial effects in improving the quality of research in clinical psychopharmacology, as well as contributing to an improvement of research and therapeutic evaluation throughout psychiatry. As a consequence, advanced research designs and methods have been adopted, especially techniques such as the double-blind placebo controlled design, quantitative methods for description of symptomatology (using rating scales and other psychometric evaluations), and multivariate statistical techniques used in conjunction with electronic computer analyses.

As an additional consequence, important information has been gathered about the drugs' modes of actions, which has generated new hypotheses about the pathogenesis of the disorder. Vigorous interchange is currently underway between clinical investigators and scientists in neurochemistry and electrophysiology. Most important, currently, are the investigations relating dopamine metabolism to the modes of actions of antischizophrenic drugs and, presumably, to impaired amine metabolism in schizophrenia.

This review will focus on the classes of compounds used in the pharmacotherapy of schizophrenia and relate their therapeutic use to currently available information about modes of action of these compounds. However, before discussing the individual compounds, a brief review of the clinical disorder is appropriate, since many readers of this series may not have had extensive clinical experience with schizophrenia.

THE CLINICAL DISORDER

Schizophrenia represents a mystery and a challenge for modern medicine. Although the phenomena of lunacy, insanity, folie, and madness have been known for centuries, modern concepts of the psychoses were formulated only in the latter half of the 19th century by Kraepelin. Kraepelin summarized most of the available descriptive psychiatry in his widely accepted textbooks which, for the first time, organized the psychiatric syndromes on an etiological basis, grouping the various disorders according to causation, i.e. infection, trauma, drug intoxication. He was then left with a large residual category of psychiatric states with no apparent etiology, and he separated this group into two forms: dementia praecox and manic depressive insanity, which he postulated were due to constitutional or metabolic causes.

Under dementia praecox, Kraepelin grouped together a number of psychotic syndromes with onset early in life and involving severe impairments of intellectual, cognitive, and perceptual functioning and gradual deterioration of social performance (5). In 1911 Bleuler, in his influential monograph, modified Kraepelin's concept by demonstrating that the nature of the thought disorder was different from classic dementias. Accordingly, he renamed the syndrome as schizophrenia (6).

Bleuler noted that most dementias occur in association with diffuse brain damage, as in the aged or following trauma, and are manifested primarily by memory defects and impairments of orientation and judgment; while in the psychopathology of schizophrenia, the predominant characteristics appear to be in disturbed associations of thought, manifested by fragmented, idiosyncratic meanings (which he called autism) and by difficulty in focusing of attention. Bleuler also demonstrated that the impairments in the regulation and expression of emotion and the profound social withdrawal, which Kraepelin had described, occur later in the clinical course, when chronicity has developed.

Currently, schizophrenia represents the most severe psychiatric disorder, not only because of its wide prevalence—it is estimated that between 1 and 3% of the adult population will experience a schizophrenic episode at some point in their life—but also because of the tendency, without modern treatment, toward recurrence and because of the high probability of chronicity with gradual and pervasive impairments in many facets of the personality. However, with pharmacotherapy and intensive efforts in psychotherapy, inpatient milieu therapy, and community mental health, there has been a modification of the pessimistic, traditional views. Much of the confusion, hopelessness, and therapeutic nihilism that have characterized professional and public attitudes about schizophrenia are presently being radically altered.

Theoretically, schizophrenia has not been established as a single disease, and until basic etiological and pathophysiological research has reached more definitive conclusions, schizophrenia must be regarded as a clinical syndrome or perhaps as a group of syndromes. It is unlikely that the group of patients currently diagnosed as schizophrenic will turn out to be a single etiological entity, but rather that subgroups will be defined with various contributions made by genetic, environmental, metabolic, and perhaps even viral etiologies. As such, it probably occupies a place in psychiatry similar to congestive heart failure in cardiology or jaundice in gastroenterology—a final common, clinical pathway resultant from multiple etiologies. Numerous hypotheses about the pathophysiology and etiology of schizophrenia have been generated, many stimulated by the recent discoveries as to the modes of action of the antipsychotic drugs. But until further research is demonstrated, the therapeutic approach to schizophrenia remains predominently empirical and clinical.

Current research on schizophrenia covers a wide range of investigations, which have been reviewed recently in a number of excellent volumes edited by Cancro (7), Romano (8), and Rosenthal & Kety (9). Etiological research focuses on possible genetic transmission, environmental influences, and biochemical abnormalities, particularly in biogenic amine metabolism and functioning (10). The diagnosis of schizophrenia cannot be validated by any specific biochemical or physiological test; it must be made on clinical criteria alone. As shown in Table 1, a range of symptoms and behaviors can be identified as characteristic of schizophrenia and used to guide clinical assessment. No single symptom or group of behaviors is in itself pathognomonic, and in making the diagnosis, the patient's symptoms and behaviors (those listed in Table 1) must be evaluated in their total pattern and understood as a configuration or complex.

For clinical purposes, the operational definition of an acute schizophrenic psychotic reaction which has been adapted for the NIMH Collaborative Phenothiazine Study can be recommended—especially where drug therapy is being considered (11). In general outline, it follows the definitions of the *American Psychiatric Association Diagnostic Manual.* Schizophrenia represents a group of syndromes or symptom states, synonymous with what was formerly called dementia praecox, which are primarily characterized by disturbances in reality testing and in concept formations. The patient's behavior may also be marked by a strong tendency to retreat from reality and by emotional disharmony, unpredictable disturbances in stream of thought, and regressive behavior. In addition, there are often affective, behavioral, and intellectual disturbances. However, the major disturbance is conceived of as lying in cognition, i.e. reality relationships and concept formation.

As outlined in Table 1, the following symptoms are characteristic of acute and chronic schizophrenic states. These symptoms may be regarded as the "target symptoms," on the basis of which patients are usually evaluated for treatment with drugs.

1. *Thinking and speech disturbances* characterized by autistic and unrealistic thinking. Depersonalization, feelings of unusual powers, perplexity, marked confusion, and dissociative phenomena are other typical manifestations.
2. *Catatonic motor behavior* characterized by conspicuous abnormal motor behavior, accompanied either by marked generalized inhibition, such as posturing, stupor, mutism, negativism, waxy flexibility, or by excessive motor activity and excitement, stereotypic behavior, and grimacing.
3. *Paranoid ideation and behavior* characterized by ideas of influence, delusions of persecution, and ideas of reference. A fairly constant attitude of hostility and aggression is often present. Excessive religiosity may be present with or without delusions of persecution. There may be an expansive delusional system of omnipotence, genius, or special ability. Patients with systematized paranoid hypochondriacal symptoms and patients who otherwise are classified as pure "paranoia" or "paranoid reaction" are usually regarded as schizophrenic.
4. *Schizophrenic hallucinations* are predominantly auditory hallucinations, occurring in absence of clinical evidence of neurological disease. Patients whose hallucinations involve sensory modalities other than the auditory sphere, i.e. visual, tactile, olfactory, should be evaluated for neurological disorders or drug reactions.

Table 1 Diagnosis of schizophrenia: characteristic symptoms and behaviors

1. Thinking or speech disturbance
2. Catatonic motor behavior
3. Paranoid ideation
4. Hallucinations
5. Delusional thinking other than paranoid
6. Blunted or inappropriate emotion
7. Disturbance of social behavior and interpersonal relations

5. *Delusional thinking* includes various forms other than paranoid, i.e. megalomanic, self-accusatory, hypochondriacal, or bodily delusions.

6. *Disturbances of affect and emotion.* The pathognomonic signs of chronic schizophrenic affective disturbance are "blunting" (or flatness) and inappropriateness. Acute schizophrenic patients frequently manifest anxiety and fear in marked degrees, often resulting in turmoil, excitement, and panic. Depression, apathy, and lethargy may also occur. These disturbed emotional manifestations infrequently occur alone, but are usually combined with confused thinking, perplexity, fear, dream states, and dissociative phenomena.

7. *Disturbances of social behavior and interpersonal relations.* This large category includes manifestations of social deterioration and changes in interpersonal relations. The specific manifestations of regression or deterioration depend upon the social background and personality of the patient. Changes in interpersonal relations are often difficult to evaluate because of their subtle quality. Symptoms such as apathy, attitudinal lethargy, indifference, social isolation, and abandonment of friends or contacts are typical manifestations of schizophrenic changes in interpersonal relations. If chronicity develops, patients frequently abandon bodily functions, such as eating, urinating, defecation, personal appearance, and cleanliness. Other symptoms of chronicity include unpredictable giggling, silly behavioral mannerisms, and loss of interest in intellectual and social areas. Aggressive behavior may be against the self, others, or property, and may be either overt or covert.

Clinical investigations have documented nonpsychotic forms—variously called latent schizophrenia, borderline states, pseudoneurotic schizophrenia, or ambulatory schizophrenia (12). Newer forms of treatment, particularly with pharmacologic agents, have reduced the proportion of the schizophrenic's time spent in hospitals, and this development plus the growth of community mental health facilities has contributed to the increasing emphasis on community treatment of schizophrenic patients. It is now possible for schizophrenic patients to live outside the institution and to sustain symptomatic remission. Vigorous treatment has also helped to counteract tendencies toward personality impairment, ego regression, and social withdrawal.

CLASSES OF COMPOUNDS USED IN PHARMACOTHERAPY OF SCHIZOPHRENIA

As shown in Tables 2, 3, and 4, there are eight classes of compounds that may be used in pharmacotherapy of schizophrenia. Of these eight groups, three are of major current usage—the phenothiazines, the thioxanthenes, and the butyrophenones (13).

Phenothiazines

The phenothiazines are the largest and the most widely used group (14). Chlorpromazine, the prototypic drug, was the first compound whose efficacy was established for schizophrenia, and it remains the reference compound. There are at least a dozen other phenothiazines available for use in the United States, but their similarities are so great that many generalizations can characterize the whole group.

Table 2 Major classes of compounds in clinical use

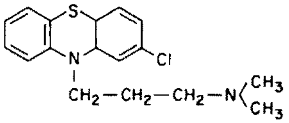
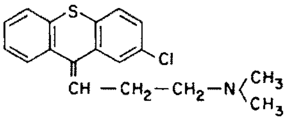
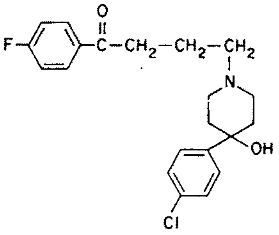
DRUG CLASS	PROTOTYPIC COMPOUND	CHEMICAL STRUCTURE
PHENOTHIAZINES	CHLORPROMAZINE (THORAZINE)	
THIOXANTHENES	CHLORPROTHIXENE (TARACTAN)	
BUTYROPHENONES	HALOPERIDOL (HALDOL)	

Table 3 Derivatives with amine-depleting actions

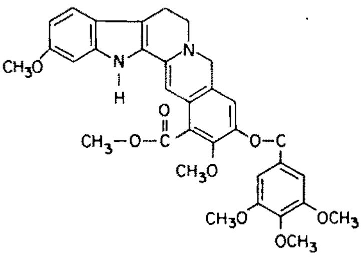
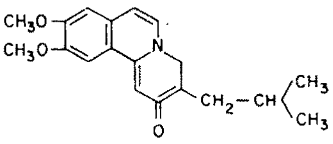
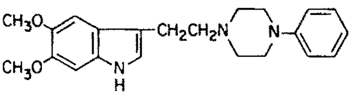
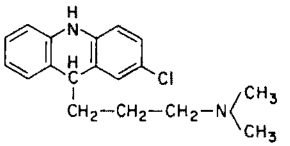
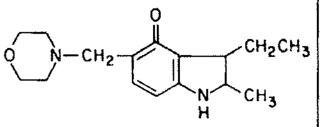
DRUG CLASS	PROTOTYPIC COMPOUND	CHEMICAL STRUCTURE
RAUWOLFIA ALKALOIDS	RESERPINE SERPASIL	
BENZOQUINOLINE	TETRABENAZINE	

Table 4 Newer compounds under investigation

DRUG CLASS	PROTOTYPIC COMPOUND	CHEMICAL STRUCTURE
PHENYLPIPERAZINES	OXYPERTINE	
ACRIDAN		
INDOLIC DERIVATIVES	MOLINDONE	

The differences among phenothiazines are related mainly to dosage, adverse effects, and clinical pharmacologic properties (15).

It is conventional to divide the phenothiazines into three subclasses, depending upon the chemical structures of the side-chains at the R-10 position. These three main subclasses are the aliphatic, the piperidine, and the piperazine derivatives, as shown in Table 5. A number of compounds are available for prescription use within each of these phenothiazine subclasses.

Compounds of the first subclass, the aliphatic subclass, have a three-carbon chain at the R-10 position. The parent compound is promazine; among the others are methoxypromazine and triflupromazine. In general, compounds in the aliphatic subclass have relatively low milligram per milligram potency. In order for patients to obtain therapeutic effects, daily dosage usually must be administered in the range of 400–600–900 mg per day. The compounds in this subclass tend to produce sedative hypnotic effects, as manifested by drowsiness, feelings of lethargy and apathy, and slowed movements. Under certain circumstances, these effects may be desirable. Chlorpromazine, particularly at 800–900 mg per day, is a very effective hypnotic agent, as well as a very effective antipsychotic agent. For example, if a patient is noisy, disruptive, agitated, or overactive, sedative hypnotic actions are appreciated by the nurses and by other patients. At night especially, a sleeping or drowsy patient is preferable to an angry disruptive one.

Patients treated with drugs from the aliphatic group experience a moderate degree of extrapyramidal symptoms. A fairly high percentage of these patients develop autonomic effects, particularly dry mouth. Constipation due to anticholinergic effects may develop, and there may also be hypotension, which requires caution when these drugs are prescribed for the elderly. Chlorpromazine, promazine, and

Table 5 Dose equivalents of phenothiazines

PHENOTHIAZINE SUB - CLASS	COMPOUND		APPROXIMATE EQUIVALENT DOSE (cpz = 100mgm)
	GENERIC NAME	COMMERCIAL NAME	
ALIPHATIC	CHLORPROMAZINE	THORAZINE	100
	PROMAZINE	SPARINE	100
	TRIFLUPROMAZINE	VESPRIN	25
PIPERIDINE	MESORIDAZINE	SERENTIL	
	PIPERACETAZINE	QUIDE	10
	THIORIDAZINE	MELLARIL	100
PIPERAZINE	ACETOPHENAZINE	TINDAL	20
	BUTAPERAZINE	REPOISE	10
	CARPHENAZINE	PROKETAZINE	25
	FLUPHENAZINE	PERMITIL PROLIXIN	1-2
	PERPHENAZINE	TRILAFON	10
	PROCHLORPERAZINE	COMPAZINE	15
	THIOPROPAZATE	DARTAL	10
	THIOPROPERAZINE	MAJEPTIL VONTIL	5-10
	TRIFLUOPERAZINE	STELAZINE	5-10

some other phenothiazines may produce liver damage and blood dyscrasias. Fortunately, these complications occur rarely, but should be considered in planning and executing a therapeutic program.

The second subclass is the piperidine derivatives. Currently, thioridazine and mesoridazine are the only piperidine compounds marketed in the United States. Mepazine was withdrawn from prescription use because it caused a relatively higher percentage of blood dyscrasias than the other compounds and also because the recommended dose range was not clinically effective. Thioridazine has considerable clinical utility as an effective antipsychotic agent in dose ranges up to 1000–1200 mg per day. However, in the higher dose range, there is danger of retinitis. Because thioridazine produces a low incidence of extrapyramidal and autonomic effects, it lends itself to use with the elderly and with outpatients for follow-up and aftercare.

There are a large number of piperazine phenothiazine derivatives. They have in common high milligram per milligram potency, i.e. 1 mg fluphenazine is the equivalent of 100 mg chlorpromazine. Potency, while an interesting pharmacologic property, is not equivalent to cost or to therapeutic effectiveness. This potency does not mean that the prescription costs only one hundredth as much; nor does it mean one hundred times more patients will be benefited with fluphenazine than with chlorpromazine. It means that the pharmaceutical chemist uses a hundredth as much in a tablet. Compared to the other phenothiazines, the piperazine derivatives are much more likely to induce extrapyramidal effects. For this reason, the piperazines have been recommended for quiet, withdrawn, and apathetic patients. However, research evidence does not entirely support this formulation.

Thioxanthenes

There are two thioxanthenes currently on the market: chlorprothixene and thiothixene (See Table 6). Their chemical structures, pharmacological actions, and range of clinical effects are very similar to the phenothiazines (16). Although the thiothixenes are becoming more widely used, they have not as yet achieved wide popularity, at least as indicated by the number of prescriptions written. Some clinicians, though, report that the thioxanthenes may have greater sedative hypnotic and mood elevating effects than the phenothiazines and, thus, advocate them for the treatment of schizo-affective states, agitated-depressive reactions, and mixed anxiety-depressive neurotic states. These claims, however, have not yet been substantiated.

Butyrophenones

The butyrophenones are the newest group of widely used antischizophrenic drugs (17, 18). They were developed in 1959 by Janssen in Belgium, and since then, a large number of derivatives have been synthesized and studied extensively in Europe (19). They are highly potent compounds and share with the Rauwolfia derivatives many clinical properties, including the potential for inducing clinical depression. Like the piperazine phenothiazines, they produce a high percentage of extrapyramidal side effects, but in contrast to the phenothiazines, they produce autonomic reactions in only a small percentage of patients. While many Europeans feel that the butyrophenones have the greatest value in severely paranoid, withdrawn, or hallucinating

Table 6 Equivalents of commonly used compounds (other than phenothiazines)

DRUG CLASS	COMPOUND		APPROXIMATE EQUIVALENT DOSE (cpz = 100mgm)
	GENERIC NAME	COMMERCIAL NAME	
THIOXANTHENES	CHLORPROTHIXENE	TARACTAN	100
	CLOPENTHIXOL	SORDINOL	20
	THIOTHIXENE	NAVANE	5
BUTYROPHENONES	HALOPERIDOL	HALDOL	2-5
	TRIDERIDOL	?	1-3
RAUWOLFIA	RESERPINE	SERPASIL	2-5

patients, debate continues on their preferential efficacy over the phenothiazines for these patients. The butyrophenones seem to have specific and highly effective actions for patients with the rare and dramatic syndrome of de la Tourettes, a pediatric disorder marked by motor tics, outbursts of explicatives, and loud cries.

Two additional classes of compounds—the Rauwolfia derivatives and the benzoquinolines—are of greater pharmacological than clinical interest. Because they have the capacity to deplete brain amines, they have had major investigational uses.

Rauwolfias

The Rauwolfia derivatives are of considerable historical and research interest (20). Their current use derives from long standing clinical experiences in Indian medicine and from subsequent isolation by Swiss pharmacologists of the active ingredients of the Indian snake roots plant—Rauwolfia serpentina. In current American psychiatric practice, the Rauwolfia derivatives are rarely prescribed, but they are also used extensively in the treatment of hypertension. If the Rauwolfias were the only antipsychotic drugs available, however, most clinicians would conclude that they constituted a major therapeutic advance. They have demonstrated effectiveness in approximately the same range of patients as do the phenothiazines, but they seem to lack the phenothiazines' therapeutic "punch." A significant side effect of the Rauwolfias is their tendency to produce depression in 10–15% of patients. This effect is best documented with the treatment of hypertension. The Rauwolfias have had an important impact on research because of their tendency to influence brain levels of amines, and research in this area has contributed greatly to our knowledge of neuropharmacology and has stimulated hypotheses about the relationships of neurochemistry to mental disorders.

Benzoquinolines

The benzoquinolines are included for chemical and pharmacologic completeness. Although tetrabenazine, the prototypic compounds of the series, has shown some clinical utility, it is not used in the United States. The benzoquinolines are mainly of interest to research pharmacologists, because they share some of the properties of reserpine, more specifically, the capacity to deplete brain amines.

Other Classes

In addition to these five established classes of compounds, mention should be made of other series: the indolic derivatives, of which molindone is the most widely studied, the phenylpiperazines, of which oxypertine is the typical representative, and the acridans, which are related chemically to the phenothiazine nucleus. Other new drugs include pimozide and clozapine, which are still under investigation.

MEGAVITAMINS For completeness's sake, mention must be made of the claims for therapeutic efficacy of very high dose members of the vitamin B series. Sometimes called "megavitamin therapy" or "orthomolecular psychiatry," these pseudoscientific pretensions have gained considerable attention in the lay press. These claims gain some apparent scientific plausibility from the transmethylation hypotheses. All the attempts at controlled research have proven negative, and pending possible future positive results, these claims must be discarded as unsubstantiated (21, 22).

THERAPEUTIC USES

General Considerations

Clinical experience indicates that the therapeutic similarities among these classes of antischizophrenic compounds are far greater than their differences. Compounds of all groups have demonstrated clinical value in the treatment of schizophrenia in its various manifestations, but none can claim any true cure of the psychoses. We do not have agents that "cure" schizophrenia, paranoia, hebephrenia, or catatonia in any sense. Thus, as described earlier, none of the available drugs do for schizophrenia what penicillin has done for CNS syphilis. The antischizophrenic drugs do, however, resemble other agents used in internal medicine, especially in therapy of hypertension, cardiac disease, or arthritis. Symptoms are suppressed, the patient's adjustment and well-being are facilitated, and the natural tendency towards remission is accelerated. Patients appear to be better, although they are not completely well (23, 24).

All of these compounds are effective in a wide range of schizophrenic clinical manifestations—acute, chronic, ambulatory, etc. In clinical practice, the most pragmatic approach is to identify the salient manifest symptoms, a method which has come to be known as the "target symptom" approach. The target symptom approach corresponds to the nature of the decision-making process employed in the treatment of psychiatric disorders. The more manifestly psychotic the patient, the more he will respond symptomatically and behaviorally to treatment. The predominant target symptoms that respond to drug treatment are overactivity, tension, hostility, combativeness, hallucination, negativism, and poor sleep. Symptoms such as lack of insight, poor judgment, impaired memory, and disorientation are less likely to improve.

Clinical experience has been supplemented by sophisticated research studies using double-blind methods, particularly the VA and NIMH Collaborative Studies which established a definitive place for the phenothiazines and related compounds. These studies settled the debates of the late 1950s about the efficacy of chlorpromazine and other compounds over placebo. They demonstrated that the phenothiazines and related compounds have clinical effects beyond placebo and milieu effects.

Acute Psychotic States

For the acutely symptomatic hospitalized schizophrenic patient, the drug effects are of major importance. It is doubtful that individual or group therapy, milieu approaches, or community mental health programs are feasible for schizophrenics without effective pharmacotherapy.

Based on results of the NIMH Collaborative Study, 95% of acutely hospitalized patients will show some discernible degree of clinical improvement within 6 to 8 weeks. During that period, about half the patients can be expected to be markedly or moderately improved. The period of hospitalization for most acutely psychotic patients is now less than three months, a marked reduction over previous practices.

Clinical practice indicates that sedative hypnotic drugs such as chlorpromazine tend to be prescribed when patients are overactive, destructive, or noisy; whereas drugs in the piperazine group tend to be prescribed for quieter or apathetic patients. The newer, more potent agents such as the thioxanthenes and butyrophenones are advocated for the chronically psychotic or apathetic patients. These differentiations have not been well substantiated, though, by carefully controlled studies. Apathetic, quiet, hallucinating, withdrawn, socially inhibited patients often do as well on phenothiazines as the overactive, combative patients. This is another piece of evidence contradicting the phenothiazine stereotype of "tranquilizing" effects. In fact, one of the surprising findings that has emerged from systematic studies is that the patterns of response to various available antischizophrenic drugs tend to be very similar.

It is now possible for intensive treatment of acute psychotic episodes of patients still in the community to obviate the necessity of hospitalization. A significant proportion of acutely schizophrenic patients need not be hospitalized. The increasing sophistication of practitioners, the availability of community psychiatric services such as day hospitals, half-way houses, and psychiatric units in general hospital settings, the skillful use of medication, and the services of public health nurses and other nonmedical therapists offer the possibility for treatment in the community when people do not require hospitalization. These practices have important implications for physicians serving in emergency units of general hospitals or as consultants to extended care facilities or institutions.

Dosage Recommendations

As shown in Tables 5 and 6, it is possible to equate the various antischizophrenic drugs with chlorpromazine on the basis of the ratio of their effective daily doses. In acute psychotic episodes, the usual daily dose for young adult males is in the range of 600–900 mg per day of chlorpromazine or its equivalent. Dosage below 300–400 mg per day is less likely to produce effects greater than that of placebo, and dosages above 1200 mg per day are not likely to produce results except in unusual instances. Thus, the effective dose range seems to be 300–1200 mg per day of chlorpromazine.

The recommended dose regimen is to increase the patient's daily total dosage within the 300–1200 mg per day range. How quickly the dose is increased depends upon the age, weight, sex, and severity of symptoms. In general, younger patients, male patients, overactive patients, and manifestly psychotic patients require higher average daily dosages. At the same time, it must be remembered that there are great individual variations in the metabolism of these drugs. Recent evidence indicates that there can be considerable individual differences in plasma levels on identical oral doses. Techniques for measuring blood levels of chlorpromazine and its metabolism are currently under investigation, and although promising findings are emerging, these methods are not yet available for routine clinical use (25).

Recent research has also questioned many of the conventional practices in the scheduling of drug doses (26). It is still common in clinical practice to prescribe antipsychotic drugs so that they are given as often as 3–6 times a day, with the total daily amount being equally divided. Moreover, the drug is often given every day of the week, and the same drug may be given without interruption for a number of years. Extensive researches in Massachusetts and in the VA system have demonstrated that these commonly employed prescription practices are unnecessarily complicated. After the first days or weeks of treatment, when the acute psychotic symptoms have been brought under control, it is desirable to provide for drug ingestion only once or at most twice a day. Single or twice daily dosages provide equal or even greater clinical benefit than multiple dose schedules, produce fewer side effects, and reduce the need for concomitant hypnotic or antiparkinsonian medication (27).

Maintenance Treatment

Most of the discussion thus far is centered on the role of pharmacotherapy in the treatment of acute psychotic reactions. However, the majority of schizophrenic patients' lives are not spent in institutions. At any one time, most schizophrenic patients are in remission and in the community. Extensive experience has documented the value of maintenance therapy with phenothiazines and other antischizophrenic drugs for ambulatory patients residing outside the institution. Such treatment will prevent relapse and rehospitalization. There are special problems with long-term maintenance therapy, but given the current situation, the relative benefits far outweigh potential difficulties.

One of the recent innovations has been the development of long acting compounds. Some of these are members of the butyrophenone group, which can be taken orally. Others are derivatives of fluphenazine. Two fluphenazine derivatives, fluphenazine enanthate and fluphenazine decanoate, are available for injectable use; their feasibility, relative safety, and long duration of action have stimulated considerable interest in their place in maintenance therapy because patients are not required to inject medication three or four times a day, but can be treated by injection once every two or three weeks.

Other Therapies

Pharmacotherapy is not prescribed in a vacuum; the patient is treated in some social context—family, outpatient clinic, general hospital, day treatment program—and there are complex interactions between the sociopsychological therapies and pharmacotherapy (28). Efforts to demonstrate the efficacy of formal individual psychotherapy have been unsuccessful (29), but the importance of intensive psychotherapeutic activity, including attention to the family and treatment setting, is widely accepted (30).

Adverse Effects

In practice, most of the adverse effects are minor and are related to the autonomic effects of the drug—postural hypotension, dry mouth, constipation, blurred vision —or to the CNS depressant effects as manifested by drowsiness and sedation.

CONVULSIONS Convulsions are the clinical manifestation of the phenothiazines' pharmacological capacity to lower the seizure threshold in animals and humans. This pharmacological action has three clinical ramifications. First, patients with pre-existing epilepsy often experience an increased frequency of overt seizures or greater seizure activity on EEG and may, consequently, require increases in their anticonvulsant drug prescription. Secondly, patients without epileptic history may develop seizures for the first time while being treated with a phenothiazine, and thirdly, alcoholics treated with phenothiazines are more likely to show seizures, particularly during acute withdrawal reactions. The best drug treatment for delirium tremens is probably high doses of intramuscular diazepoxide derivatives.

EXTRAPYRAMIDAL EFFECTS Of greater theoretical, and perhaps even practical, significance have been the effects on the extrapyramidal system. Clinically, this is manifested by three acute syndromes: parkinsonism, dystonia, and akathisia. These acute episodes are short lived, occur mostly within the first three to ten weeks of treatment, and respond to antiparkinsonian medication. Prophylactic use of antiparkinsonian medication or maintenance medication is unnecessary, and its limitations have been documented in excellently controlled studies by DiMascio (31) and the VA group (32).

Of more serious consequence is the late appearing and persistent dyskinesia, called tardive dyskinesias. This occurs late in the course of treatment, usually after two or three years of continuing treatment, and is mainly manifested in the mouth and upper torso. The pathophysiology of this syndrome is one of intensive discussion, as is its consequence. Many clinicians may be faced with the difficult dilemma of persisting with maintenance therapy for the control of distressing symptoms and behavior, while aware of running the risk of exposing the patient to this troublesome and potentially irreversible complication.

MODES OF ACTION

Having reviewed the main classes of antischizophrenic drugs and their therapeutic use, a discussion of modes of action is called for. Greater specificity and precision in terminology and communication is desirable in view of the increasing specificity of drug actions. Until very recently, it was generally agreed that the term "antipsychotic" best described these classes of drugs. "Psychosis," however, is a term that includes a range of clinical syndromes characteristic of many psychiatric disorders, of which schizophrenia is only one, though a major one. Of particular current interest is another major psychosis, mania, which occurs as a phase of the manic-depressive disorder. The recently demonstrated value of lithium in the treatment of manic psychoses suggests the value of a term more specific than antipsychotic. Therefore, it is probably more precise to consider the drugs described in this chapter as "antischizophrenic." This terminology is comparable to the labels given to antihypertensive and anticonvulsant drugs in other fields of therapeutics (33).

Unfortunately, the professional and lay press have continued to emphasize the misleading term "tranquilizer." "Tranquilizer" is a misnomer because it implies clinical utility only in those patients who are excited and, therefore, require a calming effect. Clinical and research experience has demonstrated that current drugs also have significant effects on disturbed thinking, such as occurs in schizophrenia, and that they are unlikely to significantly influence excitation, overactivity, or agitation, which does not occur in the presence of disturbed thinking.

Widespread experience with antischizophrenic drugs, or "major tranquilizers," such as the phenothiazines, distinguishes them from "minor tranquilizers," such as those of the meprobamate series and the diazepoxide derivatives. The drugs in this latter group of "minor tranquilizers" are related more closely to the sedative hypnotics, e.g. the barbiturates. The "minor tranquilizers," especially meprobamate and the diazepoxide derivatives, are of no value in schizophrenia, do not produce effects on the autonomic or extrapyramidal nervous system, and have no known effects on CNS biogenic amines. They do produce tolerance, and in some instances, dependence and even withdrawal syndromes. These properties are similar to alcohol, the barbiturates, and many other CNS depressants used as sedative hypnotics.

A separate set of semantic problems arises from communication between the Anglo-US and the French schools of psychopharmacology. Members of the French school, whose views are influential in Western European and South American psychiatry, reserve the term "tranquilizer" for the older sedative hypnotic drugs and the meprobamate and diazepoxide series. The French school uses the term "neuroleptic" for the group of drugs called antipsychotic, antischizophrenic, or major tranquilizers in the US. The French employ this term because they feel that the effectiveness of these drugs in treating psychoses is related to their capacity to influence the autonomic and extrapyramidal nervous systems. They postulate that the sine qua non of these drugs' effectiveness against psychoses derives from their actions upon the basal ganglia and subcortical centers, especially those centers that regulate the extrapyramidal system (34). Without elaborating upon the technicalities of this debate, it should be acknowledged that the situation is complex. All classes of antischizophrenic drugs produce varying degrees of extrapyramidal side effects, and moreover, biochemical investigations demonstrate common actions upon dopamine metabolism. These are promising hypotheses for further investigations on the modes of action of these drugs, and they have suggested possible biochemical mechanisms in the pathogenesis of schizophrenia.

Pharmacological Actions

The phenothiazines and other antischizophrenic compounds have a wide variety of actions. The overall result is to diminish emotional responses to stimuli, both internal and external. In addition, there is increased excitability of the neuronal membrane and some indication of inhibition of metabolic, oxidative phosphorylation, which may contribute to the decrease in neuronal excitability. These biochemical effects need to be related to pharmacological action on animal behavior, with reduction of exploratory behavior with doses that do not produce sedative hypnotic effects. In addition to conditioned avoidance reflex behavior, antischizophrenic drugs produce inhibition of intracranial self-stimulation in subcortical reward centers and reversal of amphetamine- or apomorphine-induced stereotypic behavior (35).

Effects on Extrapyramidal Nervous System and Dopamine Metabolism

As Matthysse (36) has pointed out, many effects of the phenothiazines and other series are nonspecific, that is, found in compounds that may not have therapeutic value. Therefore, efforts are underway to find modes of actions common to therapeutically effective compounds within different series; actions not found in compounds devoid of therapeutic effects. Decrease in dopamine availability at postsynaptic receptors seems the most promising hypothesis for investigation.

Most of these biological hypotheses have been derived from knowledge of the pharmacological actions of antischizophrenic drugs. Among these pharmacological activities, the ones that have gained the most attention are the blockade of catecholamine effects, particularly the postsynaptic effects on noradrenergic and dopaminergic receptors. Thus, the dopaminergic neurotransmitter has less access to the receptor probably at subcortical pathways. The phenothiazines in particular are known to influence the amygdaloid nucleus and related centers in the limbic system, with radiation of effects on the hypothalamus and the reticular activating system.

The Rauwolfia and benzoquinoline derivatives deplete CNS stores of monoamines. The phenothiazines, thioxanthenes, and butyrophenones block the postsynaptic uptake of dopamine and enhance dopamine turnover. The role of dopamine deficiency in the pathogenesis of parkinsonism is well established (37) so it seems likely that some neurological effects of antischizophrenic drugs are mediated through drugs' actions on dopaminergic systems. Whether the therapeutic effects are also related to these actions is unclear (38).

CONCLUSIONS: PHARMACOTHERAPY AND THE PATHOGENESIS OF SCHIZOPHRENIA

The demonstration of chlorpromazine's therapeutic actions in 1952–1955 was followed by the development of many other effective agents from a variety of chemical classes. Their therapeutic effects have many common features and general pattterns of pharmacotherapy have gained wide consensus. The research implications of these therapeutic actions include marked improvement in the conduct of clinical investigations and the development of new hypotheses about neuropharmacology actions, especially on brain amines.

These pharmacological actions of antischizophrenic drugs have been linked to new hypotheses of the pathogenesis of schizophrenia. The starting point for many of these hypotheses lies in the increasing strength of evidence for genetic transmission. More recent evidence in favor of the genetic influences comes from important adoption studies that allow delineation of genetic and environmental influences. Familial association has long been observed and has been buttressed by new studies of concordance rates in twins. While evidence of the importance of the genetic transmission is increasing, even the strongest supporters of the genetic factors agree that what is inherited is not the disorder per se, but some diathesis, a vulnerability to stress, which often does not become manifest until late adolescence or young adulthood.

Possible biochemical mechanisms include the transmethylation of biogenic amines, the presence of the abnormal metabolites of dopamine, creation of tryphophan derivatives with hallucinatory properties or deficiency of DBH enzyme (39). Pharmacologic research on drugs effective in schizophrenia has focused on the catecholamines as mediators of these drugs' actions and, consequently, on their possible involvement in the pathogenesis of schizophrenia (40). Although the current biological hypotheses of schizophrenia have emphasized genetics and biochemical pharmacology, it is important to incorporate into any complete theory the evidence from the neuropsychological studies of attention which have used neurophysiological techniques, particularly the evoked cortical potential.

Emphasis on biological mechanisms in pathogenesis have not diminished the importance or interest in environmental factors. If what is inherited is some diathesis or vulnerability to stress, these genetically transmitted potentials only become manifest clinically when activated by environmental or developmental stimuli, such as adolescence, personal stress, change in familiar or interpersonal relations, historical change, migration, or economic deprivation. Recent epidemiological studies have emphasized the roles of stress, poverty, migration, and selective social mobility. The importance of environmental factors derives not only from sociologic and epidemiologic factors, but also from the extensive experience with milieu therapy, which produced the hospital reforms of the 1940s and 1950s and, more recently, increased the efficacy of therapeutic community and behavioral techniques as in the "token" economies.

Thus, the experience with pharmacotherapy has greatly contributed to developing a biological-environmental theory involving a complex sequence of potentiality in the pathogenesis of schizophrenia, including genetic determinants of vulnerability, environmental precipitants of stress, and the mediating influence of neurophysiological and biochemical mechanisms.

Looking to the future, there will be increased use of these antischizophrenic drugs. Emphasis will shift farther from hospital-based treatment to community-based treatment, with greater opportunities evolving for the involvement of general physicians, internists, and pediatricians, as well as for psychiatrists in the treatment of schizophrenia. Sophistication in clinical evaluation and in research methodology is likely to grow. The need is particularly urgent to develop techniques to evaluate long-acting compounds prescribed at very high dosages, to relate clinical efficacy to differences in blood levels, and to develop knowledge about clinical pharmacology of the compounds, particularly the ways in which individuals metabolize the drug. There will be increasing emphasis on the neurophysiologic and neurochemical mechanisms mediating the actions of these drugs. The search will continue for subcortical loci of action, including the limbic system and the reticular activating pathways, and for relationships to endocrine and metabolic central mechanisms.

In the long-term future, the hope is for the emergence of more rational therapy. Current therapies are mainly symptomatic, and perhaps even palliative. While not apologizing for these limitations, one hopes for compounds that will interrupt the pathogenic sequences at earlier stages so as to render schizophrenic patients not only better, but ideally well.

Literature Cited

1. Klerman, G. L. 1973. *Drug Ther.* 3: 94–106
2. Klerman, G. L. 1973. *Drug Ther.* 3:28–32
3. Hollister, L. E. 1971. *Brain Chemistry and Mental Disease,* ed. W. M. McIssac, 303–17. New York: Plenum
4. Hollister, L. E. 1972. *N. Engl. J. Med.* 286:984–87
5. Kraepelin, E. 1918. *Dementia Praecox.* Trans. R. M. Barclay. London: Livingstone
6. Bleuler, E. 1911. *Dementia Praecox or the Group of Schizophrenics.* Trans. J. Zinkin (1950). New York: Int. Univ.
7. Cancro, R., Ed. 1970. *The Schizophrenic Reactions.* New York: Brunner-Mazel
8. Romano, J., Ed. 1967. *The Origins of Schizophrenia.* New York: Excerpta Medica
9. Rosenthal, D., Kety, S. S. 1968. *The Transmission of Schizophrenia.* New York: Pergamon
10. Kety, S. S., Matthysse, S., Eds. 1972. *Neurosci. Res. Program Bull.* 10:4
11. NIMH-PSC Collaborative Study Group 1964. *Arch. Gen. Psychiat.* 10:246–61
12. Grinker, R. R., Werble, B., Drye, R. C. 1968. *The Borderline Syndrome.* New York: Basic Books
13. Gordon, M., Ed. 1964. *Psychopharmacological Agents,* Vols. I, II. New York: Academic
14. Gordon, M. 1967. *Psychopharmacological Agents,* ed. M. Gordon, II:2–198. New York: Academic
15. Klerman, G. L. 1970. *Clinical Handbook of Psychopharmacology,* ed. A. DiMascio, R. I. Shader, 41–56. New York: Science House
16. Petersen, P. V., Nielsen, I. M. 1964. *Psychopharmacological Agents,* ed. M. Gordon, I:301–24. New York: Academic
17. DiMascio, A., Shader, R. I., Eds. 1972. *Butyrophenones in Psychiatry.* Hewlett, New York: Raven
18. Lehmann, H. E., Ban, T. A., Eds. 1964. *The Butyrophenones in Psychiatry.* Quebec: Quebec Psychopharmacolog. Res. Assoc.
19. Janssen, P. A. 1967. *Psychopharmacological Agents,* ed. M. Gordon, II:199–248. New York: Academic
20. Schlittler, E., Plummer, A. J. 1964. *Psychopharmacological Agents,* ed. M. Gordon, I:9–34. New York: Academic
21. Kline, N. S. et al 1967. *J. Am. Med. Assoc.* 200:177–78
22. Ramsay, R. A., Ban, T. A., Lehmann, H. E., Saxena, M. A., Bennett, J. 1970. *CMA J.* 102:939–42
23. Ban, T. 1968. *Psychopharmacology.* Baltimore: Williams & Wilkins
24. Klein, D. F., Davis, J. M. 1969. *Diagnosis and Drug Treatment of Psychiatric Disorder.* Baltimore: Williams & Wilkins
25. Curry, S. H. 1971. *Proc. Roy. Soc. Med.* 64:285–89
26. DiMascio, A. 1972. *Mass. J. Ment. Health* 2:25–38
27. Neu, C., DiMascio, A., Demirgian, E. 1972. *Curr. Ther. Res.* 14:246–51
28. Klerman, G. L. 1963. *J. Nerv. Ment. Dis.* 137:143–54
29. Grinspoon, L., Ewalt, J. R., Shader, R. 1967. *Int. J. Psychiat.* 4:116–28
30. May, P. R. A. 1968. *Treatment of Schizophrenia.* New York: Science House
31 DiMascio, A. 1971. *Drug Ther.* 1:23–29
32 Caffey, E. M., Hollister, L. E., Klett, D. J., Kaim, S. C. 1970. *Principles of Psychopharmacology,* ed. W. G. Clark, J. Del Guidice. New York: Academic
33 Hollister, L. E. 1972. *N. Engl. J. Med.* 286:1195–98
34 Bobon, D. P., Janssen, P. A. J., Bobon, J., Eds. 1970. *Modern Problems of Pharmacophsychiatry: The Neuroleptics.* Basel, Switz.: Karger

35. Snyder, S. H. 1972. *Arch. Gen. Psychiat.* 27:169–81
36. Matthysse, S. 1973. *Fed. Proc.* 32: 200–5
37. Costa, E., Cote, L. J., Yahr, M.D., Eds. 1966. *Biochemistry and Pharmacology of the Basal Ganglia.* Hewlett, New York: Raven
38. Matthysse, S. 1973. Schizophrenia: relationships to dopamine transmission, motor control, and feature extraction. *Neuro Sciences 3rd Study Program.* Cambridge:MIT
39. Wise, C. D., Stein, L. 1973. *Science* 181:344–47
40. Kety, S. S. 1967. *N. Engl. J. Med.* 276:325–31

Copyright 1974. All rights reserved

INFECTIOUS COMPLICATIONS OF NONALCOHOLIC DRUG ABUSE

❖7108

Donald B. Louria, M.D.
Department of Preventive Medicine & Community Health, New Jersey Medical School, Newark, New Jersey

There was virtually no interest in the medical complications of illicit drug use until the late 1960s. At that time, two groups based in New York City reported their own substantial experience and reviewed the available literature (1, 2). Since then, a welter of publications has appeared, focused primarily on the so-called overdose phenomenon and on supervening infections. In this review, I shall cover some of the more meaningful recent publications relating to infectious complications. Perhaps the most important point to make is that some established patterns of distribution of microorganisms in microbial superinfection are changing, and in addition, new types of superinfections are being reported with increasing frequency. The most prevalent infections complicating illicit drug use are listed in Table 1.

Table 1 Major infectious complications of illicit parenteral drug use

Endocarditis
Hepatitis
Embolic pneumonia
Cellulitis
Myositis
Arthritis
Osteomyelitis
Brain abscess or meningitis
Malaria
Tetanus
Upper respiratory tract acquired pneumonia

ENDOCARDITIS

In Table 2, the distribution of microorganisms from a report summarizing the experience up until 1967 is compared with a summary of 112 cases of endocarditis in heroin abusers, which was gathered by Dr. Chatrchai Watanakunakorn of the University of Cincinnati College of Medicine for a graduate education course in the spring of 1973. In percentage distribution of the major offending microorganisms, there has really been remarkably little change over the last five years. Infections due to enterococci have decreased in prevalence whereas alpha streptococci and *Pseudomonas* have assumed increasing importance.

According to Watanakunakorn, slightly more than 50% of the infections were due to *Staphylococcus aureus,* a figure somewhat lower than the 62–70% reported in three recent series (3–5). Since there is no mandatory reporting system for such cases, there can be no proper data base, and no precise conclusions about prevalence are justified. Nevertheless, the available data clearly indicate that staphylococci are the most frequently isolated etiologic agents. Enterococci, alpha streptococci, gram-negative rods, and *Candida* species each account for 7–10% of the total.

There is a great deal of disagreement concerning the frequency of tricuspid valve involvement, the figures ranging in the series cited from 28–72% (1, 3–5). The exact percentage is unimportant; what is crucial is that physicians recognize that right-sided endocarditis occurs with inordinate frequency in the heroin-using population, and that it may be manifested either as sequential septic pulmonary emboli or alternatively by fever, heart murmur, and in some cases, emboli to the spleen, kidneys, and occasionally the brain. It is also important to realize that sequential pulmonary embolization in the addict may occur as a consequence of peripheral cellulitis and thrombophlebitis in the absence of endocarditis; consequently the

Table 2 Etiological agents in endocarditis arising in narcotic addicts: a comparison between series collected in 1967[a] and 1973[b]

Agent	1967[a] (45 cases)	1973[b] (112 cases)
Staphylococcus aureus	42.2	53.1
Staphylococcus epidermidis	4.4	2.7
Pseudomonas	6.7	5.4
Other gram-negative rods	4.4	4.5
Enterococcus	20	7.2
Candida	15.4	9.0
Alpha streptococcus	2.2	9.9
Dual infection	4.4	9.0

[a] See reference 1.

[b] Collected by Dr. C. Watanakunakorn, University of Cincinnati College of Medicine. Excluded from the 1973 series are cases included in the 1967 series.

presence of multiple pulmonary emboli, although extremely suggestive, does not absolutely guarantee involvement of the tricuspid valve.

In those suffering from tricuspid valve endocarditis, at least 75% and in some series 90% of the isolated bacteria are staphylococci. In contrast, although staphylococci are frequently isolated in left-sided disease, the predominance is not nearly so marked (1, 3–6).

Several points merit emphasis. First, as emphasized by Ramsey, Gunnar & Tobin (6), in the addict both alpha streptococci and so-called nonpathogenic species of *Candida* may cause endocardial mischief even if there is no previous valvular damage. Indeed, in at least 30% of users, the evidence suggests the valve was perfectly healthy prior to invasion with these organisms.

Second, recent, and for the most part unreported, data suggest that although the overall percentage of gram-negative infections has not changed dramatically, there has been an abrupt increase in prevalence of *Pseudomonas* infections involving both the right and left side of the heart (7). Precisely why this is so is not certain, but the increasing importance of this particular organism has altered the initial chemotherapeutic approach to include antibiotics effective against both staphylococci and *Pseudomonas* (until the results of cultures are known).

Third, in at least 5% of cases, more than one organism is isolated; virtually any combination can occur, the most frequent being staphylococci and gram-negative bacilli or a bacterium plus *Candida* (1). If, therefore, a patient who is being treated for infection with a single known organism fails inexplicably to improve, the possibility of dual infection should be investigated both by obtaining new cultures and, if possible, by review of the initial culture bottles and plates.

Fourth, there are a small number of cases in which the major infection was found on the pulmonic valve (8); here again, the organisms thus far implicated are primarily staphylococci and strains of *Pseudomonas.*

Fifth, the growing evidence suggests strongly that the separation of left- and right-sided endocarditis by etiologic agent is not nearly as clear cut as previously thought. Five years ago the data showed that virtually all right-sided infections were due to coagulase-positive or -negative staphylococci (1). In late 1971 and early 1972, it became evident that strains of *Pseudomonas* could also be responsible for tricuspid or pulmonic valve infections (7, 7a). Presently, the case reports show that strains of enterobacteria, enterococcus, alpha streptococcus, and species of *Candida* may also be recovered from patients whose clinical and/or pathological findings indicate right-sided valvular disease (5).

Sixth, a small number of cases indicate that endocardial mischief is not limited to heroin abusers. Those injecting other drugs, such as stimulants, are susceptible to precisely the same microbial superinfections, including endocarditis (9, 10).

Initial treatment before culture results are known would appear to require an antibiotic insusceptible to penicillinase (such as methicillin or cephalothin) plus a drug such as gentamicin. In cases of *Pseudomonas* involvement of the tricuspid valve, the data are meager, but on the basis of one study involving five patients, the recommended approach is intensive antibiotic treatment for at least a period of several weeks followed by resection of the tricuspid valve without prosthetic replacement (7). It is still much too early to make any judgment about the long-term physiological effects of such an operation. Certainly, experimentation with this radical departure from conventional treatment is justified in view of the abysmal prognosis among addicts acquiring *Pseudomonas* endocarditis.

The majority of patients suffering from staphylococcal endocarditis recover after appropriate therapy (3, 6). In one of the earlier summaries in 1967 (1), 60% of those with staphylococcal disease survived, and this success rate has increased in the intervening years. Thus, the mortality in the cases reported in 1973 by Banks et al from Washington, DC was 16%, and in the recent series by Menda & Gorbach from Chicago, there were no fatalities in 16 cases of heroin-related staphylococcal endocarditis (3, 6).

Those parasitized by alpha streptococci or gram-negative organisms other than *Pseudomonas* also ordinarily respond adequately to therapy. The situation with *Candida* is far different. Although many cases remain unreported, it is clear that the majority of drug users acquiring candidal endocarditis will die. Dr. Nalini Premsingh of the Englewood Hospital in Englewood, New Jersey reviewed the available data in mid-1973 and found 22 adequately detailed case reports. In 12 instances no treatment was reported and all died. Four were treated with amphotericin B alone and all these also died. Six were treated both by administration of amphotericin B and by surgery; 3 of these survived. Thus, the limited evidence suggests that prompt surgery and concomitant use of amphotericin B is the regimen of choice. What role 5-fluorocytosine might play in such cases is uncertain.

Finally, Dryer & Fields reported neurologic manifestations in 14 of 28 episodes of infective endocarditis in drug abusers (4). The findings included facial paresis, hemiplegia, hemianopsia, meningoencephalitis, subarachnoid hemorrhage, nuchal rigidity, toxic encephalitis, and in a few cases, spinal cord or peripheral nerve involvement. Whether the frequency of neurologic manifestations truly differs from that in cases of endocarditis arising in non-heroin users is not certain.

HEPATITIS

There simply are no convincing data documenting the incidence of acute hepatitis, persistent hepatitis, or cirrhosis in the heroin-using population. Because heroin use is illegal, there is no known denominator on which to base the incidence of acute hepatitis. It is well documented that acute hepatitis occurs for the most part in heroin abusers in their teens or early 20s (1). Furthermore, data from the Center for Disease Control show that between 1965 and 1970, the incidence of so-called serum hepatitis increased 4-fold, whereas the increase in infectious hepatitis (hepatitis A) was far more moderate (11). The enormous increase in hepatitis B has been ascribed to the epidemic of illicit drug use (11). In 1965, Norris & Potter (12) reported that 80% of addicts dying of non-liver related causes showed evidence of hepatitis at autopsy. Marks & Chapple observed a similar prevalence of liver dysfunction in heroin-cocaine users in England (13). Cherubin and his colleagues found that 22 of 44 heroin users dying of acute narcotism or trauma in New York City showed histological evidence of hepatitis (14).

The prevalence of hepatitis in the Norris and Potter autopsy series does not necessarily reflect the incidence in the heroin-using population at large, since such series may be unintentionally extremely selective. Furthermore, the liver function test abnormalities, primarily the glutamic pyruvic and glutamic oxalacetic transaminase values, may not be a true indication of prevalence of infection with hepatitis-B viruses because these serum abnormalities could result from alcohol ingestion or other substance abuse (15).

Cherubin et al studied addicts referred to a therapeutic community and found that approximately two fifths of those studied shortly after arrival showed transaminase abnormalities (16). This does not mean that 40% of heroin users develop hepatitis-B infection. In the same study, Australia antigen was detected by the Ouchterlony technique in only 1.2% of the sample. However, this study will have to be repeated with the much more sensitive radioimmunoassay for both hepatitis-B antigen and antibody to the virus. Only then can these data, combined with the liver function studies and the histologic observations, provide a reasonable estimate of the incidence of infection and the potential for transmission to neophyte drug users. It will also be necessary to determine a demographic profile of the heroin-using population in general and of the sample to be studied to make sure that the two are comparable.

It is of course not only the heroin user who gets hepatitis. In Sweden during the mid and late 1960s, the epidemic of intravenous use of methamphetamines, phenmetrazine, and methylphenidate—all central stimulants—was accompanied by a striking increase in the prevalence of clinical cases of hepatitis (17).

It is clear that most of those injecting heroin or stimulants who acquire hepatitis will have hepatitis-B antigen in their blood during the acute phase. Using Ouchterlony double-diffusion in gel precipitin technique, immunoelectro-osmophoresis, and radioimmunoassay, Iwarson (18) found antigen in slightly less than 80% of cases. Antigen persisted for an average of 3.1 weeks after onset of illness, an interval similar to that found in non-drug-using patients with hepatitis associated with hepatitis-B antigen.

Iwarson et al (19) also studied 27 addicts who suffered multiple attacks of jaundice and found three disparate patterns. Hepatitis-B antigen was often found in serum specimens, but not during more than one attack. During other attacks, no hepatitis-B antigen was detected, but IgM levels were elevated, a pattern characteristic of short incubation period infectious hepatitis. In these two types of jaundice, biopsies showed clear evidence of viral hepatitis. In other instances, there was no hepatitis-B antigen in serum and levels of immunoglobulins were approximately normal; in these cases liver biopsy showed a nonspecific histologic reaction. Presumably, this type of jaundice was not due to either hepatitis A or hepatitis B and might be due at least in part to the direct toxic effects of the injected materials. Thus in any given drug user, jaundice occurring repeatedly could on one occasion be due to hepatitis A, on a second occasion to hepatitis B, and on a third occasion could be related to neither virus.

Iwarson concludes on the basis of very persuasive studies that about four fifths of the viral hepatitis cases in addicts are due to hepatitis B, and one fifth due to hepatitis A (18).

The other major questions relate to persistence of chemical abnormalities and the likelihood of developing chronic hepatitis, fibrosis, and cirrhosis.

Cherubin and his colleagues found that 22 of 44 heroin users dying of acute narcotism or trauma in New York City showed evidence of chronic aggressive or persistent hepatitis (14). In this study, aggressive hepatitis was characterized by cellular infiltrates, piecemeal necrosis, and septal bridging, whereas persistent hepatitis findings consisted primarily of infiltration with inflammatory cells, predominantly in the portal areas. Cherubin et al also found that 1 in 5 addicts referred to a therapeutic community had persisting transaminase abnormalities 9 months later (16). A small number of biopsies were performed in those showing continuing transaminase abnormalities; in 11 of 16, the biopsies showed either persistent or aggressive hepatitis (14).

It is again important to emphasize that the populations studied are not necessarily representative of the general heroin-using population.

Iwarson (18) studied a group of blood donors in Sweden. Six stimulant habituees were found to have hepatitis-B antigen in serum specimens. Three of these developed clinical hepatitis and subsequently the serum became negative for antigen. The other 3 had demonstrable antigen for more than 4 months; in all 3 transaminase values were elevated and biopsies showed persistent hepatitis. There is some as yet preliminary data suggesting that persistent antigenemia with subtype *ay* is more likely to be associated with persistent or aggressive hepatitis on biopsy than persistent antigenemia with hepatitis-B subtype *ad* (18).

Tamburro, Rajan & Leevy of the New Jersey Medical School reported a small number of addicts who showed fibrosis or cirrhosis on liver biopsy (20). In a few cases, serial biopsies showed acute hepatitis followed by fibrosis and then by lobular distortion. The relative roles of hepatitis viruses, malnutrition, and alcohol remain to be clarified.

The only valid conclusion would seem to be that some heroin abusers show histological abnormalities consistent with continuing hepatitis-B infection, and in some cases this may progress to fibrosis and cirrhosis. Even this judgment must be tempered in that some of the abnormalities found on biopsy may be due not to the effects of a hepatitis virus but rather to the influence of alcohol or other drugs being used by the heroin abuser (15, 18).

If there is persistence of transaminase elevations or persistence of hepatitis-B antigens, the likelihood of chronic or aggressive hepatitis on biopsy is great. There is no evidence that the presence of antibody to hepatitis-B antigen is associated with any hepatic dysfunction or histologic abnormality (18).

It will obviously take another decade or more to determine how frequently the hepatitis observed in intravenous users of illicit drugs leads to chronic disease and/or cirrhosis, and what influences that frequency.

CENTRAL NERVOUS SYSTEM

Infections of the nervous system have not in the past been included in reviews of the microbial complications of heroin use, but there is growing evidence, although thus far anecdotal, indicating that yeast or fungal infections of the central nervous system may supervene in the parenteral drug user, presumably related to the egregiously unhygienic techniques used in the injecting of heroin. There are no data suggesting increased susceptibility to or frequency of bacterial infections of the central nervous system.

Three cases of focal mucormycotic involvement of the brain, each fatal, in otherwise healthy young heroin habituees have been reported (21–23). Additionally, there is one instance of lethal central nervous system aspergillosis in a 37-year-old heroin addict who had no other underlying disease (24).

Recently, we have observed a patient who used heroin and subsequently developed indolent meningitis. No organisms were recovered from the cerebrospinal fluid using conventional media for recovery of bacteria and fungi. Because no organism was isolated, serum was studied for *Candida* precipitins and these were found to be present in high titer. *Candida albicans* was then isolated from the spinal fluid in hypertonic sucrose media but not in conventional isotonic media.

In these five cases, none of the determinants associated with *Candida, Aspergillus,* or mucor infections of the nervous system was present. There are so few cases of microbial infection of the brain due to these organisms in the uncompromised host that the assumption must be made that the injection of heroin played the major role in introducing those organisms into the blood stream, thus permitting subsequent seeding of the nervous system. It would seem virtually certain that cases of yeast and fungal meningitis and brain abscess will be reported with increasing frequency so long as intravenous drug abuse persists at a significant level in our society.

LUNGS

Septic embolization to the lung occurs secondary to tricuspid or pulmonic valve infection, but in addition, severe embolic pneumonia can result from infections of the extremities consequent to injection of various materials in and around veins (1, 25). In addition, there is some reason to believe that the use of heroin is associated with a greater incidence of upper respiratory tract acquired bacterial pneumonia (1, 26). In two reports, the organisms implicated were predominantly pneumococci and the infections responded promptly to proper antibiotic therapy (1, 26). Unfortunately, no adequate epidemiologic studies have been carried out so that it is unclear whether upper respiratory tract acquired pneumococcal infection is in actuality more frequent in the heroin abuser. Additionally, there are no convincing data indicating the possible mechanisms of such alleged susceptibility. We have performed studies on peritoneal mobilization of polymorphonuclear leukocytes in heroin-treated and control mice; there was no evidence that opiate-induced stupor impaired polymorphonuclear mobilization. It would appear likely that, as with alcohol, if heroin predisposes to upper respiratory tract acquired bacterial pneumonia, it does so primarily by vitiating glottis closure.

SKIN, SUBCUTANEOUS TISSUES, AND MUSCLE

It is of course well known that local injection of heroin and other drugs into the skin and subcutaneous tissues either intentionally or inadvertently while attempting to inject substances into the veins promotes local infections. These may be due to a variety of organisms, including staphylococci, β-hemolytic streptococci, and gram-negative rods such as strains of *Pseudomonas, Serratia,* and *Klebsiella.* Particularly with β-streptococcal infection, the local cellulitis and abscesses may be accompanied by demonstrable bacteremia. Lewis has noted more severe pyomyositis and necrotizing fasciitis in drug abusers (27) Here again staphylococci predominate, but β-streptococci and anaerobic streptococci have also been implicated. In such cases, the most effective treatment consists of antibiotics accompanied by surgical débridement. We have also seen occasional cases of *Candida* myositis. Interestingly, the myositis and fasciitis may be found involving sites of the extremities considerably distant from the sites of drug injection (27).

Of the drugs being used illicitly by injection, those most likely to induce local inflammation are the barbiturates. Injection of these drugs intramuscularly or subcutaneously characteristically produces tenderness, induration, fibrosis, and contractures (28).

The most feared local infection is of course tetanus. Only small numbers of such cases are reported each year, almost exclusively in so-called skin-poppers who inject narcotics, stimulants, or barbiturates subcutaneously. This results in the occurrence of interconnecting abscesses that provide an ideal milieu for the growth of *Clostridium tetani.* This complication occurs at least as frequently or perhaps more frequently in female addicts (1), despite the fact that the male to female ratio in the addict population is 4–6:1. Why women should have a proclivity to develop tetanus is uncertain. It may be that women have not been in the armed forces and therefore are less likely to have been immunized against tetanus. An equally plausible explanation is that for currently arcane reasons at least as many women as men show a willingness to inject heroin subcutaneously if they have used up the available veins in their arms and legs.

If tetanus supervenes in an addict, the mortality remains greater than 50% despite prompt therapy (29). Treatment is similar to that in the general population except that it may be more difficult to extirpate or adequately drain the extensive local lesions. A recent report suggests that early débridement may increase the likelihood of convulsions and death (30); nevertheless, débridement is still advocated by most authorities although whether infiltrating immune globulin into the local lesion in addition to administering it systemically is beneficial remains unsettled.

Tetanus is now seen infrequently enough in the addict that physicians are beginning to fail to make the diagnosis promptly; even risus sardonicus can be overlooked if the physician is not attuned to considering this diagnosis in the drug-using population.

RENAL

There is little direct evidence of microbial infection of the kidneys in parenteral drug users. Chmel & Grieco reported one instance of renal colic occurring in a 20-year-old man who also suffered from diabetes (21). Subsequently, multiple *Aspergillus fumigatus* fungus balls were passed, these apparently arising from the right renal pelvis.

A small number of cases of nephrotic syndrome have occurred in addicts (31). In these cases, kidney biopsies showed proliferative glomerulonephritis with deposition predominantly of IgM rather than IgG. There was no evidence that the renal lesions were conventional post-streptococcal glomerulonephritis. Whether this represents an allergic response to heroin-related microbial parasitism or whether it represents an immunologic response to another injected substance is not known. It is likewise unclear how frequently this complication supervenes and what course it ordinarily takes.

BONES AND JOINTS

A few years ago, heroin-related arthritis or osteomyelitis was virtually undescribed. This has changed dramatically in the last two years. Arthritis may be found in the large joints of the extremities, or in the sternoclavicular, achromioclavicular, or sacroiliac joints (32, 33). In cases of osteomyelitis, a variety of sites may be involved, including the vertebral column, clavicle, symphysis pubis, and ischeal tuberosity (33–36). Currently, the organism isolated most frequently is *Pseudomonas aeruginosa* with *Staphylococcus aureus* a close second. In a few cases of joint or bone involvement, *Candida* species or gram-negative organisms other than *Pseudomonas* have been isolated.

MISCELLANEOUS PROBLEMS

Malaria

In the 1930s, hundreds of cases of lethal needle-induced falciparum malaria were reported (37, 38). This problem disappeared in the 1940s, perhaps because of the widespread use of quinine as an adulterant. Malaria surfaced again when malaria-infected Vietnam war veterans returned to this country and became part of the domestic drug scene (39). Thus far, the number of mini-epidemics is small and the infecting organism has been *Plasmodium vivax.* Transmitted in this manner, there is no exoerythrocytic phase and chloroquine alone effects a predictable cure. It seems likely that eventually the more malignant falciparum variety will also be seen in the United States in intravenous drug users.

Fever With Negative Blood Cultures

Often after the injection of heroin, fever supervenes, ranging up to 104–105° F. Blood cultures are sterile and there is no accompanying leukopenia. Ordinarily, the fever disappears within 48 hr; currently its precise cause remains obscure. Fever may also occur from certain drugs themselves; for example, in one case an oral overdose of methamphetamine resulted in profound hyperpyrexia and reversible renal failure (40).

Sepsis Without A Focus

Intravenous drug users may develop transient or sustained bacteremia in the absence of any demonstrable focus. In fact, the drugs are prepared with such total disregard for sterility that habituees almost certainly experience frequent bacteremia. This may account at least in part for the elevation of heat-stable opsonins observed in the addict population (41). The most impressive incidence of sepsis occurred in Detroit in the early 1960s when the heroin-using population switched to the more easily obtainable paregoric. When injected, paregoric scleroses veins and soon the paregoric users were injecting the drug directly into the jugular vein. Frequent infections were inevitable; in one 9-month period, about 3% of the known addicts were admitted to one hospital with presumed bacterial infections including sepsis (42).

Serological Studies In Addicts

It has long been known that heroin abuse may be associated with the presence of false-positive tests for syphilis. Cherubin and his colleagues have investigated the serological response to a variety of antigens in addicts and controls. Up to 20% of heroin addicts showed significant antibody titers to *Lymphogranuloma venereum* and Q fever antigens (43). This was a far higher percentage than found in the controls. In most cases, the titers were low, but in some cases they were high enough to potentially mislead the clinician into thinking active infection was present. The addicts also showed a greater prevalence of Proteus OX19 antibodies and positive tests for mononucleosis (44, 45). Their mean titer of antibody to typhoid, paratyphoid, and *Brucella* antigens was also significantly greater than observed in controls (45).

It has been assumed that these positive tests reflect a nonspecific elevation in IgM and IgG, but the studies thus far carried out do not define this relationship adequately. There is at present no evidence that the presence of this vigorous antibody response is deleterious to the addicts.

MECHANISMS OF INCREASED SUSCEPTIBILITY TO INFECTION

Much of the increased incidence of infection in the addict certainly results from putting organisms directly into the blood stream or establishing local lesions in the skin, subcutaneous tissues, veins, or muscles that form the nidus for severe focal or systemic infections.

Heroin addicts possess increased amounts of heat-stable opsonins that are effective in promoting phagocytosis of certain gram-negative bacteria and *Staphylococcus aureus* (41). This increased opsonic capacity appears to reside in both IgM and IgG antibodies, but does not seem to either protect the addict from microbial superinfection or exert undesirable effects.

Narcotics do not appear to vitiate the ability of human polymorphonuclear leukocytes to either ingest or kill microorganisms (41). As noted above, we found no evidence that heroin reduces the mobilization of polymorphonuclear leukocytes in an experimental animal model. Thus, there are no data indicating that the addict suffers from an abnormality of polymorphonuclear leukocyte function. It has been suggested that the addict may suffer from defects in delayed immune mechanisms mediated by lymphocytes and macrophages. There are thus far no convincing data to support such a notion. It may well be that the injection of microbes and non-living foreign materials may, by inducing leukocytosis, cause an ephemeral diminution in lymphocyte function and skin reactivity (46), but currently this intriguing phenomenon has no known clinical significance.

PREVENTION OF SUPERINFECTION

Aside from convincing intravenous drug users to abstain or inject in proper hygienic fashion, there would seem to be no practical technique for obviating infection, but in a few cases, primary prevention should at least be attempted. This is well illustrated by the following case:

A 17-year-old black boy was admitted to the Martland Medical Center in Newark, New Jersey in June 1973 because of fever, shortness of breath, and chest pain of 7-days duration. He had suffered from rheumatic fever, and at age 14 the mitral valve had been replaced by a prosthesis. For the last 6 months he had used heroin regularly. On admission, the only finding specifically suggestive of endocarditis was splenomegaly. Chest X ray was consistent with either congestion or multiple emboli. Initial therapy for presumed infection on the prosthetic valve consisted of gentamicin and cephalothin. Fever persisted and petechiae appeared as did evidence of emboli to the head. By the 5th hospital day, all blood cultures showed a species of *Candida* and treatment with amphotericin B was started. Over the next five days, relentless deterioration ensued despite the addition of 5-fluorocytosine (to which the organism was subsequently found to be resistant). Replacement of the prosthesis was planned, but on the 10th hospital day, heart block supervened and the patient died. At autopsy, massive candidal vegetations were found on the prosthesis; the tricuspid valve was normal.

Perhaps earlier surgery might have helped but whatever the therapeutic approach at that stage with that particular organism, the prognosis was bleak. Data from the narcotic registry maintained in my Department suggest that in the prime target group, black Newark males age 20–30, 1 in 7 is a repeated heroin user. Thus, the epidemiologic evidence clearly indicates how vulnerable this patient was in terms of likelihood of heroin use as a growing man. The most sensible approach would have been for the physicians to sit down with the patient and his parents and older siblings at age 14 and explain the immense peril of intravenous drug use in an individual with a prosthetic heart valve. Preventive educational efforts in the drug scene have not been encouraging; nevertheless, this would have been the proper approach to any young man with valvular heart disease who lives in an area of such extraordinary endemic parenteral drug use. In this case, involvement of older siblings might have served to put additional pressure on the patient. At any rate, in selected patients, intensive efforts to achieve primary prevention, that is avoidance of risk factors, should surely be attempted.

Literature Cited

1. Louria, D. B., Hensle, T., Rose, J. 1967. The major medical complications of heroin addiction. *Ann. Intern. Med.* 67: 1–22
2. Cherubin, C. E. 1967. The medical sequellae of narcotic addiction. *Ann. Intern. Med.* 67:23–33
3. Menda, K. B., Gorbach, S. L. 1973. Favorable experiences with bacterial endocarditis in heroin addicts. *Ann. Intern. Med.* 78:25–32
4. Dryer, N. P., Fields, B. N. 1973. Heroin-associated infective endocarditis. *Ann. Intern. Med.* 78:699–702
5. Banks, T., Fletcher, R., Ali, N. 1973. Infective endocarditis in heroin addicts. *Am. J. Med.* 55:444–51
6. Ramsey, R. G., Gunnar, R. M., Tobin, J. R. Jr. 1970. Endocarditis in the drug addict. *Am. J. Cardiol.* 25:608–18
7. Arbula, A., Thoms, N. W., Wilson, R. F. 1972. Valvulectomy without prosthetic replacement. A lifesaving operation for tricuspid pseudomonas endocarditis. *J. Thorac. Cardiov. Surg.* 64:103–7

7a. Reyes, M. P., Palutke, W. A., Wylin, R. F., Lerner, A. M. 1973. Pseudomonas in the Detroit Medical Center 1969–1972. *Medicine* 52:173–94

8. Massumi, R. A., Just, H., Tawakkol, A. 1967. Pulmonary valve regurgitation secondary to bacterial endocarditis in heroin addicts. *Am. Heart J.* 73:308–16
9. Wickramasekaran, R., Banks, T. 1972. Amphetamine endocarditis. *N. Engl. J. Med.* 287:1251
10. Hahn, H. H., Schweid, A. I., Beaty, H. N. 1969. Complications of injecting dissolved methylphenidate tablets. *Arch. Intern. Med.* 123:656–59
11. Gregg, M. B. 1972. The changing epidemiology of viral hepatitis in the United States. *Am. J. Dis. Child.* 123: 350–54
12. Norris, R. F., Potter, H. P. Jr. 1965. Hepatic inflammation in narcotic addicts. *Arch. Environ. Health* 11:662–68
13. Marks, V., Chapple, P. A. L. 1967. Hepatic dysfunction in heroin and cocaine users. *Brit. J. Addict.* 62:189–95
14. Cherubin, C. E. et al 1972. Chronic liver disease in asymptomatic narcotic addicts. *Ann. Intern. Med.* 76:391–95
15. Stimmel, B., Vernace, S., Tobias, H. 1972. Hepatic dysfunction in heroin addicts. The role of alcohol. *J. Am. Med. Assoc.* 222:811–12
16. Cherubin, C. E., Kane, S., Weinberger, D. R., Wolfe, E., McGinn, T. 1972. Persistence of transaminase abnormalities in former drug users. *Ann. Intern. Med.* 76:385–89
17. Iwarson, S. 1969. Somatiska Komplikationer vid narkotikamissbruk (4): Infekioner och hepatiter. *Lakartidningen* 66:1986–2006
18. Iwarson, S. 1963. Studies on viral hepatitis and hepatitis B (Australia) antigen. *Scand. J. Infec. Dis. Suppl.* 7:5–30
19. Iwarson, S., Ludin, P., Holmgren, J., Hermodsson, S. 1973. Multiple attacks of hepatitis in drug addicts: Biochemical, immunochemical and morphological characteristics. *J. Infec. Dis.* 127:544–50
20. Tamburro, C. H., Rajan, K. S., Leevy, C. M. 1971. Development of cirrhosis following hepatitis. *Gastroenterology* 60: 167
21. Chmel, H., Grieco, M. H. 1973. Cerebral mucormycosis and renal aspergillosis in heroin addicts without endocarditis. *Am. J. Med. Sci.* 266:225–31
22. Hameroff, S. B., Eckholdt, J. W., Lindenberg, R. 1970. Cerebral phycomycosis in a heroin addict. *Neurology* 20:261–65
23. Adelman, L. S., Aronson, S. M. 1969. The neuropathologic complications of narcotics addiction. *Bull. N. Y. Acad. Med.* 45:225–34
24. Burston, J., Blackwood, W. A. 1963. A case of aspergillus infection of the brain. *J. Pathol. Bacteriol.* 86:225–29
25. Briggs, J. H., McKerron, C. G., Souhami, R. L., Taylor, D. J. E., Andrews, H. 1967. Severe systemic infections complicating in "mainline" heroin addiction. *Lancet* II:1227–31
26. Cherubin, C. E. 1971. Infectious disease problems of narcotic addicts. *Arch. Intern. Med.* 128:309–13
27. Lewis, R. J. 1973. Infections in heroin addicts. *J. Am. Med. Assoc.* 223: 1036–37
28. Pollard, R. 1973. Surgical implications of some types of drug dependence. *Brit. Med. J.* 1:784–87
29. Skudder, P. A., Inglis, A. E., McCarroll, J. R. 1962. Tetanus in New York City. *N. Y. State J. Med.* 62: 2793–2800
30. Ayim, E. N. 1972. The management of tetanus—A study of 51 cases treated at Muhimbili Hospital, Dar Es Salam. *East Afr. Med. J.* 49:170–76
31. Kilcoyne, M. et al 1972. Nephrotic syndrome in heroin addicts. *Lancet* I:17–20
32. Tindel, J. R., Crowder, J. G. 1971. Septic arthritis due to Pseudomonas

aeruginosa. *J. Am. Med. Assoc.* 218: 559–61

33. Madhavan, T., Salahuddin, N., Fisher, E., Quinn, E. 1972. *Pseudomonas osteomyelitis in young narcotic addicts.* Presented at 12th Interscience Conf. Antimicrob. Agents and Chemother., Atlantic City, N. J., Sept. 26–29
34. Holzman, R. S., Bishko, F. 1971. Osteomyelitis in heroin addicts. *Ann. Intern. Med.* 75:693–96
35. Fishbach, R. S., Dahlgren, J. G., Rosenblatt, J. E. 1972. *Vertebral osteomyelitis due to gram negative bacteria.* Presented at 12th Interscience Conf. Antimicrob. Agents and Chemother., Atlantic City, N. J., Sept. 26–29
36. Lewis, R. J., Gorbach, S., Altner, P. 1972. Spinal pseudomonas chondroosteomyelitis in heroin users. *N. Engl. J. Med.* 286:1303
37. Most, H. 1940. Falciparum malaria among drug addicts. Epidemiologic studies. *Am. J. Pub. Health* 30:403–10
38. Helpern, M. 1934. Epidemic of fatal estivoautumnal malaria among drug addicts in New York City transmitted by common use of hypodermic syringe. *Am. J. Surg.* 26:111–23
39. Malaria Surveillance. Center for Disease Control. 1971. Ann. Rep., U.S. Dept. HEW, Atlanta, Georgia, June 1972
40. Ginsberg, M. D., Hertzman, M., Schmidt-Nowara, W. W. 1970. Amphetamine intoxication with coagulopathy, hyperthermia and reversible renal failure. *Ann. Intern. Med.* 73:81–85
41. Nickerson, D. S., Williams, R. C. Jr. Boxmeyer, M., Quie, P. G. 1970. Increased opsonic capacity of serum in chronic heroin addiction. *Ann. Intern. Med.* 72:671–77
42. Oerther, F. J., Goodman, J. L., Lerner, A. M. 1964. Infections in paregoric addicts. *J. Am. Med. Assoc.* 190:683–86
43. Cherubin, C. E., Millian, S. J. 1968. Serologic investigations in narcotic addicts I. Syphylis, Lymphogranuloma, Venereum, Herpes simplex and Q fever. *Ann. Intern. Med.* 69:739–42
44. Millian, S. J., Cherubin, C. E. 1971. Serologic investigations in narcotic addicts III. Latex fixation, C-reactive protein, "monotest", serum proteins and SH antigen. *Am. J. Clin. Pathol.* 56:693–98
45. Vogel, H., Cherubin, C. E., Millian, S. J. 1970. Febrile agglutinins in narcotic addicts. *Am. J. Clin. Pathol.* 53:932–35
46. Heiss, L. I., Palmer, D. L. Anergy in patients with leucocytosis. *Am. J. Med.* In press

Copyright 1974. All rights reserved

ROLE OF GASTRIN IN HYPERSECRETORY DISORDERS IN MAN

❖7109

James E. McGuigan, M.D.
Division of Gastroenterology, Department of Medicine, University of Florida, College of Medicine, Gainesville, Florida

INTRODUCTION

Secretion of hydrochloric acid by the stomach has captured the attention and research efforts of some of the most distinguished investigators in the history of gastrointestinal physiology. Enormous interest in hydrochloric acid secretion by the stomach has been retained both because of the extraordinary sequence of physiological events that permits the stomach to concentrate hydrogen ions by more than one millionfold in gastric juice and also because of the direct clinical implications of abnormalities in rates of gastric acid secretion. A variety of common, unusual, and rare clinical syndromes and diseases are associated with disturbances in rates of acid secretion by the stomach. In several of these diseases disordered gastric acid secretion appears to be of crucial importance in the initiation and/or perpetuation of the pathological process. The precise mechanism(s) by which acid secretion participates in the pathogenesis of some of these diseases, e.g. peptic ulcer, remains to be clarified in completeness. It is in relation to the control of gastric acid secretion that we direct our attention to a substance about which an enormous amount of information has been recently accumulated and which appears to play a most important role in stimulating gastric acid secretion—the polypeptide hormone gastrin.

GASTRIN

Gastrin is by far the most potent agent known in stimulating acid secretion by the stomach. (Histamine remains a distant second.) It was by its capacity to evoke gastric acid secretion that gastrin was first (1) identified and characterized in gastric mucosal extracts by John Edkins in the earliest years of the present century. However, just as with most other polypeptide hormones, gastrin possesses multiple physiological activities that may be expressed both on various portions of the gastrointestinal tract and on various endocrine organs (2, 3). Gastric acid secretion has been viewed as the primary, and certainly physiological, action of gastrin. It has not been established with certainty to what extent others of the multiple activities of gastrin represent pharmacological events and which may be of importance in normal physiology. It is in respect to the capacity of gastrin to stimulate gastric acid secretion that this discussion is directed.

In 1964 Gregory, Tracy and colleagues (4–7) successfully purified and characterized gastrin from porcine antral mucosa, and were successful in the synthesis of the hormone. They identified and described two gastrin species, each of which contained 17 amino acids in a single polypeptide chain (heptadecapeptides), which they named Gastrin I and Gastrin II. They identified the amino acid sequence of these heptadecapeptides, and indicated that Gastrin II differed from Gastrin I only by the sulfation of the tyrosine residue in position twelve in Gastrin II. Subsequently the amino acid sequences of gastrin heptadecapeptides from various other mammalian species, including man (8), were identified. The human gastrin heptadecapeptides were found to be identical to those originally purified from hog antral mucosa, except for the substitution of leucine in human gastrin for the methionine residue in position five in porcine gastrin. The amino acid sequence of human Gastrin I is ⌊Glu-Gly-Pro-Try-Leu-(Glu)$_5$-Ala-Tyr-Gly-Try-Met-Asp-Phe-NH$_2$. Availability of pure naturally occurring gastrins, as well, subsequently, as synthetic human gastrin, paved the way for the development of a variety of radioimmunoassay techniques (9–16), which has made possible the sensitive and specific measurement of gastrin in the serum, plasma, and tissue extracts (17–30).

Although the major form of gastrin in gastric antral mucosa is that of heptadecapeptide gastrin, in 1970 Yalow & Berson (31) demonstrated that the major form of gastrin in the circulation is a larger species of gastrin, which they termed "big gastrin." Big gastrin was also found to exhibit a less marked net negative charge than heptadecapeptide gastrin, which, by virtue of its abundance of dicarboxylic amino acids, possesses an extremely strong negative electrical charge: for this reason big gastrin has also been referred to as "basic gastrin." Big gastrin has been shown to contain 33 amino acid residues, in contrast to the 17 contained in heptadecapeptide gastrin (32). Incubation of big gastrin with trypsin yields two basic peptides and a strongly acidic peptide with the amino acid composition of heptadecapeptide gastrin and possessing all of the physiological activities of known heptadecapeptide gastrins (33). It is reasonable to conclude that trypsin digestion of big (or basic) gastrin cleaves a peptide bond between lysine and the amino-terminal residue of heptadecapeptide gastrin, yielding a gastrin in the heptadecapeptide amide form. The calculated minimum molecular weight for big gastrin is approximately 3870 compared with approximately 2200 for heptadecapeptide gastrin. The molecular weight of big gastrin has been estimated, on the basis of Sephadex G-50 filtration, to be approximately 7000 (31). The discrepancy between this value and the chemically determined value (mol wt 3870) may be explained by either anomalous behavior of big gastrin on Sephadex (a phenomenon observed with reasonable frequency) or the possibility that big gastrin may exist in dimeric form. There are experimental data (32) in support of the view that big gastrin, just as heptadecapeptide gastrin, exists in both sulfated and nonsulfated forms (big gastrins I and II). There is evidence that the time of onset and the continuation of activity in stimulating gastric acid secretion is more prolonged with big gastrin than with heptadecapeptide gastrin (34). The precise relationships between big gastrin and heptadecapeptide gastrin, i.e. whether they are synthesized separately or represent a precursor and product relationship, have not yet been clarified.

Recently, Yalow & Berson identified an additional species of gastrin that is larger in size than big gastrin, and have designated this material as "big, big" gastrin (35). Big, big gastrin was identified in extracts of human postmortem proximal jejunum, with big, big gastrin comprising from 6–24% of immunoreactive gastrin in the jejunal extracts. Antral extracts did not contain significant quantities of big, big gastrin. Following incubation with trypsin, 85% of big, big gastrin appeared to be converted to a heptadecapeptide-like component. Big, big gastrin exhibits electrophoretic mobility on starch gel electrophoresis just in advance of serum albumin, i.e. with mobility that is not noticeably different from big gastrin (36). Big, big gastrin, which had been eluted from Sephadex G-50 in the void volume, was found by ultracentrifugal analysis to have a sedimentation velocity similar to human growth hormone (mol wt 20,000). The biological significance of big, big gastrin has not yet been evaluated.

THE ZOLLINGER-ELLISON SYNDROME

In 1955 Zollinger & Ellison (37) described the syndrome which to this day, in familiar usage, bears their name: the classic triad that constitutes this syndrome is comprised of severe ulcer disease of the upper gastrointestinal tract, marked gastric acid hypersecretion, and the presence of non-beta, non-alpha islet cell tumors of the pancreas. Other investigators (38, 39) had noted the association of non-insulin-producing islet cell tumors with gastric hypersecretion and intractable peptic ulceration before the observations of Zollinger & Ellison; however, Zollinger & Ellison were the first to suggest that a hormone contained in and released from these tumors was responsible for the development of the clinical manifestations of this disorder. Time and efforts of other investigators have established the wisdom and accuracy of their proposal (40–44). It has now been established beyond reasonable doubt that the polypeptide gastrointestinal hormone gastrin is present in abundant quantities in the tumors (42–44) and in the circulation (43) of patients with the Zollinger-Ellison syndrome, and that the pathophysiological events that characterize the Zollinger-Ellison syndrome represent consequences of excess circulating gastrin. It has been proposed that, in analogy to insulin-containing insulinomas, the term "gastrinoma" be applied to Zollinger-Ellison tumors.

The true incidence of the Zollinger-Ellison syndrome in the general population has not been established, nor is the true frequency of Zollinger-Ellison tumors among patients with peptic ulcer disease known. By 1964 (45), 260 cases had been recorded in the literature and the number now exceeds 1000. It has been estimated that peptic ulcer disease occurs in from 10–15% of the general population and thereby represents a disease of enormous frequency and importance. It is probable that less than 1% (and how much less is uncertain) of patients with the diagnosis of peptic ulcer harbor tumors of the Zollinger-Ellison variety. The majority of patients with the Zollinger-Ellison syndrome in the presently recorded literature probably constitute those with more flagrant forms of the disease. With increasing availability of gastrin radioimmunoassay more subtle manifestations of the Zollinger-Ellison syndrome will be identified, its frequency will be established, and this frequency will almost surely exceed that suggested previously in the biomedical literature.

Prior to the development and application of gastrin radioimmunoassay, measurement of gastric acid secretion provided the most reliable diagnostic method for suspecting and supporting the diagnosis of the Zollinger-Ellison syndrome: further verification of the diagnosis awaited histological documentation at surgery or postmortem examination. It is true that patients with Zollinger-Ellison tumors usually have basal gastric acid hypersecretion. Criteria that have been suggested for gastric acid secretory rates consistent with the diagnosis of Zollinger-Ellison tumors are a basal gastric acid secretion exceeding 15 meq HCl/hr (46) and/or a 12-hr gastric acid secretion greater than 100 meq HCl (45). A ratio of basal acid to maximum histamine- (or histalog-) stimulated gastric acid secretion greater than 0.6 is found in approximately 60% of patients with Zollinger-Ellison tumors: this ratio is usually substantially smaller in normal individuals and in patients with common peptic ulcer disease (46–48). The variability of rates of gastric acid secretion among patients with Zollinger-Ellison tumors and their overlap with those of normal individuals and patients with non-Zollinger-Ellison peptic ulcer disease prohibit the reliable use of estimations of gastric acid secretion in diagnosing the Zollinger-Ellison syndrome. Identification of gastric acid hypersecretion in a patient with peptic ulcer disease, however, now requires serum or plasma gastrin measurement by radioimmunoassay to establish or exclude the diagnosis of the Zollinger-Ellison syndrome.

The clinical features of the Zollinger-Ellison syndrome, in its presently recognized state, have been well established. Abdominal pain, the most common symptom (45, 49, 50), is present in almost all patients with the Zollinger-Ellison syndrome and is frequently similar to that experienced by patients with non-Zollinger-Ellison peptic ulcer disease; however, it should be stressed that the pain is frequently unusually persistent and/or severe. There is no significant sex predominance (45). The diagnosis of the Zollinger-Ellison syndrome is usually established in the third to fifth decades, but has been made in children as young as seven years of age (51) and in patients as old as ninety (52).

In a review of 260 cases (45), 93% were found to have peptic ulcer disease, with the remaining 7% having diarrhea without peptic ulcer. Diarrhea, which is present in from one third to three quarters of patients with the Zollinger-Ellison syndrome, is responsive to treatment by gastric aspiration as well as by total gastrectomy (53–56). The diarrhea in patients with Zollinger-Ellison tumors is due principally to the presence of excessive gastric acid secreted into the upper gastrointestinal tract. Elevated concentrations of circulating gastrin, by direct effects on the bowel, may in some instances contribute to the diarrhea of Zollinger-Ellison patients. Administration of pentagastrin (which contains the carboxyl-terminal tetrapeptide amide active portion of gastrin) has been shown to reduce the absorption of water in the human proximal small intestine (57). Furthermore, in three patients with the Zollinger-Ellison syndrome (two of whom had total gastrectomy), jejunal absorption of water, glucose, and sodium was shown to be reduced (58). The responsiveness of the diarrhea in Zollinger-Ellison patients following either gastric aspiration or total gastrectomy suggests that direct hormonal effects of gastrin are not major factors responsible for the diarrhea frequently found in Zollinger-Ellison patients.

Abnormal histologic features (59, 60) have been identified in the small intestinal mucosa of Zollinger-Ellision patients with gastric acid hypersecretion. These have included gastric metaplasia of the jejunal mucosa, submucosal edema and hemorrhage with microerosions, abnormal villous architecture, and cellular infiltration of the lamina propria. Steatorrhea is frequently found among Zollinger-Ellison patients; however, the precise frequency of this abnormality has not been established. Evidence has been provided that steatorrhea with Zollinger-Ellison tumors is a consequence of inactivation of pancreatic lipase at low pH within the intestinal lumen as a result of excess gastric acid hypersecretion (59, 60); pancreatic lipase is irreversibly denatured by acid. In addition, bile salts (especially those conjugated to glycine) are precipitated at low pH, thereby reducing their availability for micelle formation, and consequently decreasing intraluminal digestion of lipid (61). Vitamin B_{12} malabsorption (59, 62) has also been identified among Zollinger-Ellison patients; however, the mechanism by which this occurs is not known.

Zollinger-Ellison gastrinomas frequently occur as components of the multiple endocrine adenoma syndrome (Type I). Pituitary abnormalities (adenoma, carcinoma, or hyperplasia) were reported in at least 10% of Zollinger-Ellison patients by Ellison & Wilson (45) and in 4 of 12 patients by Friesen (63). Tumors or hyperplasia of other endocrine organs are found in as many as 45% of patients with Zollinger-Ellison gastrinomas, with hyperparathyroidism being the most frequent abnormality (45). Thyroid, ovarian, and adrenal abnormalities are less common. Endocrine dysfunction in association with the multiple endocrine adenoma syndrome may be the result of hyperplasia, adenomas, or carcinoma. The genetic transmission of Type I multiple endocrine adenoma syndrome is that of autosomal dominance with variable expressivity. In a review by Ballard et al (64) of patients with Type I multiple endocrine adenoma syndrome, 88% had parathyroid abnormalities with pancreatic and pituitary abnormalities in 85% and 65% respectively. A 19% incidence was found for adrenal abnormalities and also for thyroid dysfunction. Rarely, an islet cell tumor may produce multiple hormones (65–67): such tumors have been shown to contain and release, in addition to gastrin, adrenocorticotrophic hormone, melanocyte-stimulating hormone, glucagon, and insulin.

The Zollinger-Ellison syndrome also may result from adenomas, carcinomas, or hyperplasia of non-beta, non-alpha islet cells arising within the pancreas or, less commonly, from the duodenal mucosa. These islet cells have been designated as non-beta and non-alpha both because of their morphological and histochemical characteristics and since, except in rare instances, they contain neither insulin nor glucagon. It has seemed enigmatic that Zollinger-Ellison tumors should arise within the pancreas since the major residence of gastrin is the mucosa of the stomach and upper small intestine. It has not been possible to demonstrate conclusively gastrin activity (biological activity) in pancreatic extracts. However, with immunohistochemical techniques (68, 69) success has been achieved in demonstrating gastrin in islet cells of the normal human pancreas. Utilizing fluoroscein-labeled antibodies to gastrin (69), gastrin has been demonstrated in granules packing the cytoplasm of islet cells located principally in the periphery of human pancreatic islets. These cells have the staining characteristics of delta cells. It is probable that these, or related, cells represent the cells of origin of Zollinger-Ellison tumors. Although almost all Zollinger-Ellison tumors arise within the pancreas or duodenal mucosa, one instance of the Zollinger-Ellison syndrome due to an infiltrating carcinoma of the stomach (70), which was shown by immunofluorescence to contain gastrin, has been reported. In spite of the fact that the gastric antral mucosa is the major location for gastrin, the phenomenon of a Zollinger-Ellison tumor arising in the stomach must be extraordinarily rare.

Polak, Stagg & Pearse (71) have suggested the possible existence of two types of Zollinger-Ellison syndrome. One group of Zollinger-Ellison patients had markedly elevated serum gastrin levels with profound antral gastrin cell hyperplasia with relatively short clinical history of disease. The second group of Zollinger-Ellison patients, with longer histories, had somewhat lower serum gastrin concentrations, absence of antral gastrin cell hyperplasia, but the presence of either pancreatic gastrinomas or hyperplasia of pancreatic delta cells.

The factors responsible for the emergence or expression of Zollinger-Ellison gastrinomas have not been identified. Several authors have suggested that primary hypothalamic stimulation, with resultant excessive activity of the anterior pituitary, may be the initiating event (63, 71, 72). In support of this hypothesis, which has by no means been proven, the following observations are submitted for consideration. Gastric acid secretion both in experimental animals and man has been shown to be reduced in association with anterior pituitary insufficiency (73). It has been demonstrated that the inhibition of gastrin release in experimental animals following hypophysectomy was corrected following administration of growth hormone (74). In patients with acromegaly, gastric acid secretion is increased and gastrin cell hyperplasia occurs in the gastric mucosa (75). Friesen et al (63, 72) have suggested a pituitary influence on pancreatic islet cells and endocrine polypeptide neoplasms. Polak, Stagg & Pearse (71), following concepts developed by Friesen (63, 72), have suggested the following as a possible mechanism for development of Zollinger-Ellison gastrinomas. The Zollinger-Ellison syndrome may be initiated by a primary pituitary influence stimulating antral gastrin cell hyperplasia with resultant increased gastric acid secretion. Acid secreted by the stomach produces inhibition of gastrin release. This antral inhibition produces a "gastric factor" that further stimulates the pituitary to produce the agent responsible for stimulation of gastrin cell hyperplasia. This pituitary agent in turn stimulates the synthesis and release of gastrin by cells other than those in the antral mucosa, including pluripotential cells in the islets of Langerhans or duodenal mucosa with the resultant development of Zollinger-Ellison gastrinomas or hyperplasia.

By light microscopy with hematoxylin and eosin staining, Zollinger-Ellison gastrinomas exhibit a remarkable variety of cytological architectural patterns (76). The cytoplasm of individual tumor cells varies in amount from scanty to abundant and in staining characteristics from acidophilic to basophilic. Gastrinoma cells are usually round or polygonal, occasionally ovoid, and rarely spindle-shaped. Although nuclei are usually round and uniform, in some instances they are pleomorphic with bizarre nuclear forms. Multinucleated gastrinoma cells are rarely seen. Mitotic figures in individual tumors vary from none to numerous, with atypical mitoses extremely rare, even among obviously malignant tumors. Some gastrinomas are highly cellular, and others may contain only thin rows of tumor cells widely separated by abundant collagen. Foci of necrosis are commonly found in metastases of gastrinomas, but rarely in the primary tumors. Glandular structures are identified in approximately one third of gastrinomas, and are more frequent (75%) in duodenal gastrinomas where glandular components are often prominent. There are no morphological characteristics that distinguish Zollinger-Ellison gastrinomas from non-beta islet cell tumors associated with the pancreatic cholera syndrome (to be discussed below). Gastrin has been identified in the cytoplasm of gastrinoma tumor cells utilizing immunofluorescent techniques (71, 75, 76). In a study of six Zollinger-Ellison gastrinomas (76), large amounts of gastrin, ranging from 1050 to 12,500 pg/ml tissue, were identified in the tumors. By electron microscopy (76) Zollinger-Ellison gastrinoma tumor cells have been shown to contain cytoplasmic granules of moderate to high electron density, each of which is surrounded by a limiting membrane. Two populations of gastrinoma tumor cells have been identified on the basis of the size and appearance of their granules; one cell population contains round homogenous granules (diameter range from 150–200 nm) and the other contains pleomorphic granules (diameter range up to 350 nm). In two thirds of gastrinomas the former cell population predominates. Similar to light microscopy, at the electron microscopic level no distinction can be made between gastrinoma tumor cells and tumor cells of the pancreatic cholera syndrome.

In most patients with the Zollinger-Ellison syndrome, the predominant form of circulating gastrin is that of big gastrin (31, 32). However, it should be noted that the ratio of circulating gastrin, which is heptadecapeptide gastrin, to that which is big gastrin is highly variable from patient to patient (31, 33). Yalow & Berson (35) recently noted that a very small proportion of circulating immunoreactive gastrin (0.9–2.6%) exhibits the characteristics of big, big gastrin.

In contrast to circulating immunoreactive gastrin (mostly big gastrin), tumor gastrin, just as antral gastrin, usually exists predominantly in the form of heptadecapeptide gastrin. Recently, utilizing radioimmunoassay, starch gel electrophoresis, and Sephadex chromatography (77), 4 of 5 Zollinger-Ellison tumors were found to contain 83% or more of immunoreactive gastrin as the heptadecapeptide. These observations are in agreement with those of Gregory & Tracy who have found, utilizing bioassay techniques for measurement of gastrin activity, that from two thirds to 90% of gastrin activity in pooled extracts from gastrinomas conforms with heptadecapeptide gastrin (32, 34). It has been shown that even in individual patients with gastrinomas, in whom the major circulating gastrin species is big gastrin, that the major hormone form in the tumor is heptadecapeptide gastrin (77). Grossman and colleagues (78) have recently shown a substantially shorter half-life for heptadecapeptide gastrin than for big gastrin: the longer biological half-life of big gastrin when compared with the heptadecapeptide may explain the relative preponderance of big gastrin in the circulation when compared with its proportionately smaller content in Zollinger-Ellison tumors.

Interesting relationships have been identified between gastrin and calcium, particularly in patients with the Zollinger-Ellison syndrome (79–81). It has been shown that in response to constant intravenous calcium infusion large amounts of gastrin, presumably of tumor origin, are released into the circulation in patients with Zollinger-Ellison tumors. The amount of gastrin released in patients with gastrinomas substantially exceeds that observed in normal individuals and in patients with common duodenal ulcer following calcium infusion. The calcium challenge test is performed by constant intravenous infusion of calcium gluconate for a 3-hr period at the rate of 5 mg calcium/hr: serum samples for gastrin radioimmunoassay are obtained prior to initiation of calcium infusion and at 30-min intervals for 4 hr. The maximal gastrin response is usually achieved in the interval between 2 and 3 hr following the initiation of calcium infusion. In Zollinger-Ellison patients there is, in general, an excellent correlation between the increases in circulating calcium and gastrin levels in response to calcium infusion. In response to calcium infusion not only is there excessive gastrin release in patients with gastrinomas (increment usually > 500 pg gastrin/ml), but their acid secretory responses to calcium infusion are also greater than those of normals and patients with common duodenal ulcer. Thus the calcium infusion test constitutes an excellent provacative test in identifying patients with the Zollinger-Ellison syndrome, particularly those without marked hypergastrinemia, i.e. with fasting serum gastrin concentrations in the range from 200–600 pg/ml.

It has recently been shown that gastrin and pentagastrin possess the capacity to stimulate calcitonin release from the thyroid gland (82–84). In clinical analogy, patients with gastrinomas with hypergastrinemia have been found frequently to have increased blood levels of calcitonin (85). It has been suggested, and appears most reasonable to conclude, that the mechanism for elevated calcitonin levels in Zollinger-Ellison patients is that increased calcitonin release is a consequence of increased circulating gastrin concentrations. It should be indicated, however, that the possibility, though less likely, of coexistent calcitonin-releasing tumors in Zollinger-Ellison patients has not been excluded.

A second and more recently described provocative test for identifying patients with gastrinomas is that of secretin infusion (24, 86, 87). Secretin, in man, decreases gastric acid secretion, probably by inhibition of both gastrin-mediated gastric acid secretion and gastrin release. In normals and in patients with common duodenal ulcer, intravenous infusion of secretin lowers serum gastrin concentrations (20, 88). In contrast, intravenous infusion of secretin in patients with gastrinomas has been shown to produce a paradoxical increase in serum gastrin concentrations (24, 86, 87). The mechanism by which secretin raises serum gastrin concentrations in Zollinger-Ellison patients and the proportion of patients with gastrinomas in which this phenomenon may be induced have not been established. An increase of serum gastrin concentrations in Zollinger-Ellison patients has been observed both with constant intravenous infusion of secretin (86) and with a single intravenous secretin injection (81). The magnitude of increase in serum gastrin concentration appears to be greater and more reliable with constant intravenous infusion of secretin, which is probably the preferred method when this test is applied in an attempt to substantiate the diagnosis of the Zollinger-Ellison syndrome.

Thus, a profile of serum gastrin concentrations, which consists of the following, may be described for Zollinger-Ellison patients: 1. elevated fasting serum gastrin concentrations in a patient who usually demonstrates gastric acid hypersecretion, 2. exaggerated gastrin release in response to calcium infusion, 3. the frequently observed paradoxical increase in serum gastrin concentrations following intravenous secretin infusion.

In a few patients with Zollinger-Ellison gastrinomas following total gastrectomy, primary tumors and/or pre-existent metastatic tumor, at the time of subsequent surgical re-exploration, have been found to be substantially decreased in size or to have disappeared (72, 98). The mechanism by which these events occur and the frequency of this phenomenon following total gastrectomy in patients with gastrinomas are not known. Serum gastrin concentrations and the clinical course of 6 patients with metastatic Zollinger-Ellison gastrinomas following total gastrectomy have been described (72). In 2 of the 6 patients serum gastrin concentrations following gastrectomy returned to normal levels, and in these patients no tumor was found at "second look" operations. In 2 others in this group of 6 patients serum gastrin concentrations following total gastrectomy remained moderately elevated in the Zollinger-Ellison range: with surgical re-exploration in these patients, 3–9 years following gastrectomy, tumor persisted, although with evidence of partial regression. In the final 2 patients serum gastrin concentrations progressively increased following total gastrectomy, with resultant marked hypergastrinemia (4150–50,000 pg/ml); at subsequent surgery these patients had evidence of progression of their disease, with increasing size of metastases, and both patients expired secondary to their disease. The data from these patients suggest that postoperative serum gastrin concentrations may be of some value in identifying regression of Zollinger-Ellison tumor and perhaps in identifying patients with progressive tumor advancement.

THE PANCREATIC CHOLERA SYNDROME

The pancreatic cholera syndrome (89–92) is discussed here to provide distinction between this entity and the Zollinger-Ellison syndrome. As previously indicated, the Zollinger-Ellison syndrome is characterized by gastric acid hypersecretion and is frequently accompanied by diarrhea which in some instance is voluminous: the pathophysiological events observed in Zollinger-Ellison patients represent direct consequences of excess gastrin release. The pancreatic cholera syndrome [synonyms include Verner-Morrison (89) syndrome and the WDHA (watery diarrhea, hypokalemia, achlorhydria) (92) syndrome] is also associated with non-beta and non-alpha islets of tumors of the pancreas, and is characterized by frequent, often overwhelming, diarrhea with water and electrolyte losses which may be of life-threatening proportions. Unlike the Zollinger-Ellison syndrome, pancreatic cholera is not associated with increased gastric acid secretion—acid secretory rates are either normal or decreased. [In some instances (90) these patients have been achlorhydric.] Gastrin concentrations are not elevated in the sera of patients with pancreatic cholera syndrome nor is gastrin found in extracts of these tumors (76). It is believed that pancreatic cholera results from release of a hormone into the circulation which increases intestinal losses of water and electrolytes and has the capacity to inhibit gastric acid secretion. Following surgical removal of these islet cell tumors the diarrhea disappears, and in those previously achlorhydric patients gastric acid secretion returns to normal. Various hormones have been suggested as potential etiological agents in this abnormality. Among others, the candidate hormones have included secretin (93), prostaglandins (94), gastrin plus glucagon (95), gastric inhibitory peptide (GIP) (96), and vasoinhibitory peptide (VIP) (97). In spite of these proposals, the hormone responsible for the pancreatic cholera syndrome, although not gastrin, has not been established.

GASTRIN AND DUODENAL ULCER

Duodenal ulcer is the major and most important form of peptic ulcer disease. As a group, patients with duodenal ulcer have increased rates of gastric acid secretion when compared with control subjects of similar age and sex (99, 100). It should be pointed out, however, that not all patients with duodenal ulcer disease have gastric acid hypersecretion. It is now evident that some patients with duodenal ulcer have rates of acid secretion which are markedly elevated, and may even be indistinguishable from those of patients with Zollinger-Ellison gastrinomas. Inasmuch as gastrin plays a crucial role in the regulation of gastric acid secretion, attention has been directed to the possible role of gastrin in common duodenal ulcer. Unlike the Zollinger-Ellison syndrome, in spite of occasionally similar rates of gastric acid secretion, duodenal ulcer is not associated with increased fasting serum gastrin concentrations (16, 21, 29). In most studies fasting serum and plasma gastrin levels do not differ from those of control subjects. Acid within the gastric lumen constitutes a potent inhibitor of gastrin release. Since gastrin levels in duodenal ulcer patients, frequently with gastric hypersecretion, are not lower than those of normal control subjects, it has been postulated (101) that the normal feedback control mechanism by which low antral pH inhibits gastrin release may be deficient in patients with duodenal ulcer. Studies by several groups of investigators (29, 102, 103) have indicated that in response to a protein-containing meal, a known potent stimulus to gastrin release, the amount of gastrin released into the circulation in duodenal ulcer patients is greater than that observed in nonulcer subjects. An important related question in the comparison of duodenal ulcer patients with normal individuals is whether, in respect to their gastric acid secretory responses, the former are more sensitive to given doses or concentrations of gastrin. It has been recently shown that patients with duodenal ulcer were more sensitive to various administered doses of pentagastrin (which contains the physiologically active portion of the gastrin molecule) and secreted more acid in response to pentagastrin than patients without duodenal ulcer (104). Thus, in spite of the absence of fasting hypergastrinemia, it would appear that gastrin may be of importance in the hypersecretory state which frequently characterizes duodenal ulcer.

Using bioassay techniques, Emas and associates (105) found that the antral gastrin content was similar in patients with duodenal ulcer and in normal subjects. In contrast, they found that patients with gastric ulcer had lower than normal antral gastrin activity. Somewhat in contrast to the above observations, but using different techniques, other workers (71, 106) have found evidence by immunofluorescence for increased numbers of gastrin-containing cells, perhaps with increased cytoplasmic content of gastrin, in the antral mucosa of patients with duodenal ulcer disease. Accurate quantitative information concerning the antral gastrin content of duodenal ulcer patients remains to be acquired.

GASTRIN AND GASTRIC ULCER

Unlike duodenal ulcer gastric ulcer is not associated with increased rates of gastric acid secretion: acid secretory rates in patients with gastric ulcer (107) are usually within the normal range or are decreased. Antral gastrin activity (as cited above) has been found to be reduced in gastric ulcer patients when compared with both duodenal ulcer and normal subjects (105). Unlike duodenal ulcer patients fasting serum gastrin concentrations in patients with gastric ulcer have been found to be slightly increased (21). It is probable that in this instance increased gastrin levels reflect reduced rates of acid secretion, and thereby, reduction of the mechanism that inhibits gastrin release into the circulation. In these patients increases in serum gastrin concentration were correlated with decreases in rates of gastric acid secretion. These observations would not support the prior suggestion that gastric stasis participates in the pathogenesis of gastric ulcer by stimulation of gastrin release. Presently available information does not provide support for an important role for gastrin in the production of gastric ulcer.

GASTRIN FOLLOWING PEPTIC ULCER SURGERY

Present surgical procedures for the treatment of peptic ulcer disease are directed at reductions in rates of gastric acid secretion. The major current duodenal ulcer operations include antrectomy with vagotomy or pyloroplasty with vagotomy; vagotomy may be performed as bilateral truncal vagotomy or selective vagotomy, in which vagal branches that innervate the stomach are selectively transected, or proximal (also referred to as superselective or parietal cell) vagotomy, in which those vagal fibers innervating the acid-secreting portion of the stomach are interrupted. Following vagotomy and antrectomy, circulating gastrin levels are significantly reduced (26, 31), but not to immeasurable levels, indicating that immunoreactive gastrin from sources other than the antrum remains in the circulation. Extra antral sources that may contribute to circulating gastrin concentrations following antrectomy include gastrin from the mucosa of the upper small intestine and perhaps from the pancreas. In spite of the marked decreases in acid secretion by the stomach that follow pyloroplasty and vagotomy, serum gastrin concentrations are not reduced following these procedures; in fact, in patients following vagotomy and pyloroplasty serum gastrin levels are often higher than those obtained preoperatively (23, 26, 103). This observation indicates that the reduction in gastric acid secretion that follows vagotomy and pyloroplasty is not the result of decreases in circulating serum gastrin levels. Alternatively, it appears much more reasonable to suggest that the principal effects of vagotomy and pyloroplasty in reducing gastric acid secretion are those that result from removal of vagal activity by adjusting (raising) the acid secretory threshold for gastrin and/or from removal of direct effects of vagal stimulation on acid-secreting parietal cells.

Insulin-induced hypoglycemia evokes vagally stimulated gastric acid secretion, resulting in part from promotion of gastrin release. There is some difference of opinion as to whether the gastrin response to insulin is abolished (108) or only diminished (109) following vagotomy. Fasting and postprandial serum gastrin concentrations in duodenal ulcer patients treated surgically with partial gastrectomy and either gastroduodenal or gastrojejunal anastamosis (103) have indicated that gastrin levels were reduced to a greater degree in those patients with gastrojejunal anastamosis than in those with gastroduodenal approximations. These differences were interpreted as indicating greater gastrin release in patients with gastroduodenal anastamosis, thus providing evidence for release of duodenal gastrin in this group, which was not observed in the patients with gastrojejunal anastamosis. [Duodenal mucosa (110) contains from one third to almost as much gastrin as is contained in the mucosa of the antrum of the stomach.]

HYPERPARATHYROIDISM, PEPTIC ULCER, AND GASTRIN

The most clearly established relationship between gastrin and hypersecretion of gastric acid and its pathophysiological consequences is the gastrinoma of the Zollinger-Ellison syndrome. As previously stated, hyperparathyroidism frequently occurs with gastrinomas (45). The effects of hypercalcemia in stimulating both excess gastrin release and gastric acid secretion in patients with hyperparathyroidism (but without gastrinomas) and the relationship between hyperparathyroidism and peptic ulcer disease are less clearly understood. There is evidence in support of an increased prevalence of peptic ulcer disease among patients with hyperparathyroidism (111–113). However, when patients with either clinically evident or inapparent Zollinger-Ellison tumors are excluded there is controversy as to whether there remains an increased incidence of peptic ulcer among hyperparathyroid patients. Normal levels of serum calcium appear necessary for basal gastric acid secretion. It has been noted that when serum calcium concentrations are less than 7 mg%, e.g. in hypoparathyroidism, acid secretion is abolished; however, with return to normal calcium levels, gastric acid secretion returns (114). In normal individuals, intravenous calcium infusions sufficient to raise serum calcium concentrations by 3 mg% produce significant increases in secretion of both gastric hydrochloric acid and pepsin (115). Blood levels of gastrin have been found to be elevated (usually slightly or only marginally) in some, but not most, patients with hyperparathyroidism (116, 117): marked hypergastrinemia, as associated with gastrinomas, is not a characteristic of isolated hyperparathyroidism. In 18 patients with hyperparathyroidism (117), the mean fasting serum gastrin concentration was found to be 122 ± 39 SEM pg/ml (normal range 50-200 pg/ml), with 8 of these patients having values considered to be elevated or in the upper limits of the normal range. We have studied a patient with hyperparathyroidism, hypercalcemia, marked gastric acid hypersecretion, and a marginally elevated serum gastrin concentration (200 pg/ml). Following parathyroidectomy, with removal of three hyperplastic parathyroid glands, ulcer healing occurred, gastric acid hypersecretion disappeared, serum calcium levels returned to normal, and the serum gastrin level fell to 50 pg/ml. These observations (118) and those of other investigators (117) provide evidence that in some patients with hyperparathyroidism and peptic ulcer disease, calcium may induce increased gastrin release with resultant gastric acid hypersecretion, which may play a part in the pathogenesis of their peptic ulcer disease. Surgical therapeutic approach to such patients should be directed to the hyperparathyroidism: the diagnosis of the Zollinger-Ellison syndrome in such patients should be considered only if hypergastrinemia persists following correction of hypercalcemia.

GASTRIN AND CATECHOLAMINES

There is information based on studies (119) in animals and man that catecholamines possess the capacity to stimulate release of gastrin and, probably, resultant gastric acid secretion. Moderately increased fasting serum gastrin concentrations (450 and 400 pg/ml) have been reported in two patients with pheochromocytoma. Serum gastrin concentrations in these patients fell to normal following the administration of phenoxybenzamine, returning to prior elevated levels following discontinuance of the drug. Fasting serum gastrin concentrations decreased to normal following surgical excision of the pheochromocytomas. The administration of epinephrine, in doses of 4 μg/kg/min, produced significant increases in serum gastrin concentrations in dogs. Although the mechanism by which catecholamines promote release of gastrin into the circulation has not been defined, a potential mechanism for stimulation of gastrin release under these circumstances may be catecholamine-induced increases in blood flow to the gastric mucosa. It is not known to what degree, if any, catecholamines stimulate or otherwise modify release of gastrin into the circulation of normal individuals or individuals with peptic ulcer.

GASTRIN AND THE EXCLUDED ANTRUM

The excluded antrum syndrome (120, 121) is a rare entity, which may be similar to and have to be distinguished from the Zollinger-Ellison syndrome. This disorder consists of stomal peptic ulceration, usually with gastric acid hypersecretion, which occurs at some point in time following partial gastric resection, in which a portion of the gastric antrum has been permitted to remain and is removed from contact with acid secreted by the stomach (usually gastrojejunal anastomosis). It is believed that acid hypersecretion and stomal ulceration in such patients result from gastrin release from the remaining antral mucosa, which is deprived of contact with acid, the normal major mechanism for inhibition of release of gastrin. In the retained antrum syndrome the proportion of basal acid secreted to that with maximum histamine or histolog stimulation may mimic that of patients with gastrinomas. A patient with retained antrum syndrome has been reported (121) in whom the serum gastrin concentration, which was moderately elevated, decreased following intravenous secretin administration (as occurs in normals and patients with common peptic ulcer). This is in contrast to the frequently observed increases in serum gastrin concentrations following secretin infusion in patients with gastrinomas. Thus, the secretin challenge test may be of some diagnostic assistance in identifying patients with the retained antrum syndrome and distinguishing these patients from those with gastrinomas.

GASTRIN AND ACID SECRETION: ADDITIONAL CONSIDERATIONS

Understanding of the mechanisms involved in, and the location(s) of degradation and disposal of, gastrin molecules is incomplete. There is, however, accumulating evidence (122–124) that the small intestine and the kidney both play major roles in gastrin degradation and/or disposal. Patients with renal failure (125, 126) have been shown frequently to exhibit hypergastrinemia. Patients with marked renal insufficiency, as reflected by substantial serum creatinine elevations, are more likely to exhibit hypergastrinemia. Some such patients have elevated serum gastrin levels in range of those found in patients with gastrinomas. On the basis of presently available studies it is reasonable to conclude that the major mechanism for elevated serum gastrin levels in these patients is that of reduced inactivation of gastrin. (The kidney does not appear to serve as a major route for gastrin excretion.) There is no present evidence that hypergastrinemia with renal failure causes gastric acid hypersecretion, nor that it is responsible for any known gastrointestinal anbormalities.

In some patients massive intestinal resection has been associated with peptic ulcer and marked increases in gastric acid secretion. The small intestine of dogs has been shown to be responsible for a substantial proportion of inactivation of the gastrin molecule (124). It is possible, although not established, that gastric acid hypersecretion following massive intestinal resection may represent the result of gastrin-induced gastric acid hypersecretion due to reduced metabolic degradation of gastrin secondary to removal of a substantial portion of the small intestine.

Literature Cited

1. Edkins, J. S. 1905. *Proc. Roy. Soc. London* 76:376
2. Grossman, M. I. 1970. *Nature* 228: 1147–50
3. Grossman, M. I. 1970. *Lancet* 1: 1088–89
4. Gregory, R. A., Tracy, H. J. 1964. *Gut* 5:103–7
5. Gregory, H., Hardy, P. M., Jones, D. S., Kenner, G. W., Sheppard, R. C. 1964. *Nature* 204:931–33
6. Anderson, J. C. et al 1964. *Nature* 204.933–34
7. Morley, J. S., Tracy, H. J., Gregory, R. A. 1965. *Nature* 2-7:1356–59
8. Bentley, P. H., Kenner, G. W.,

Sheppard, R. C. 1966. *Nature* 209: 583–85
9. McGuigan, J. E. 1968. *Gastroenterology* 54:1005–11
10. Stremple, J. F., Meade, R. C. 1968. *Surgery* 64:165–74
11. McGuigan, J. E. 1969. *Gastroenterology* 56:429–38
12. Hansky, J., Cain, M. D. 1969. *Lancet* 2:1388–90
13. McGuigan, J. E., Trudeau, W. L. 1970. *Gastroenterology* 58:139–50
14. Yalow, R. S., Berson, S. A. 1970. *Gastroenterology* 58:1–14
15. Ganguli, P. C., Hutner, W. M. 1972. *J. Physiol.* 220:499–510
16. Trudeau, W. L., McGuigan, J. E. 1970. *Gastroenterology* 59:6–12
17. McGuigan, J. E., Trudeau, W. L. 1970. *N. Engl. J. Med.* 282:358–61
18. Reeder, D. D., Ban, J. L., Thompson, J. C. 1970. *Surg. Forum* 21:290–91
19. Ganguli, P. C., Cullen, D. R., Irvine, W. J. 1971. *Lancet* 1:155–58
20. Hansky, J., Soveny, C., Korman, M. G. 1971. *Gastroenterology* 6:62–68
21. Trudeau, W. L., McGuigan, J. E. 1971. *N. Engl. J. Med.* 284:408–12
22. Korman, M. G., Soveny, C., Hansky, J. 1971. *Gut* 12:899–902
23. Korman, M. G., Hansky, J., Scott, P. R. 1972. *Gut* 13:39–42
24. Bradley, E. L., Galambos, J. T. 1972. *Lancet* 1:594
25. Jackson, B. M., Reeder, D. D., Thompson, J. C. 1972. *Am. J. Surg.* 123:137–42
26. McGuigan, J. E., Trudeau, W. L. 1972. *N. Engl. J. Med.* 286:184–88
27. Jaffe, B. M., Peskin, G., Kaplan, E. L. 1972. *Cancer* 29:694–700
28. Thompson, J. C., Reeder, D. D., Buchanan, H. H. 1972. *Am. J. Surg.* 124:250–60
29. McGuigan, J. E., Trudeau, W. L. 1972. *N. Engl. J. Med.* 288:64–66
30. McGuigan, J. E., Trudeau, W. L. 1973. *Gastroenterology* 64:22–25
31. Yalow, R. S., Berson, S. A. 1970. *Gastroenterology* 58:609–15
32. Gregory, R. A., Tracy, H. J. 1972. *Lancet* 2:797–99
33. Yalow, R. S., Berson, S. A. 1971. *Gastroenterology* 60:202–14
34. Gregory, R. A., Tracy, H. J. 1973. *Mt. Sinai J. Med.* 40:359–64
35. Yalow, R. S., Berson, S. A. 1972. *Biochem. Biophys. Res. Commun.* 48:391–95
36. Yalow, R. S., Wu, N. 1973. *Gastroenterology* 65:19–27
37. Zollinger, R. A., Ellison, E. H. 1955. *Ann. Surg.* 142:709–28
38. Rabinovitch, J., Achs, S. 1945. *Arch. Pathol.* 40:74–77
39. Forty, F., Barrett, G. M. 1952. *Brit. J. Surg.* 40:60–63
40. Gregory, R. A., Tracy, H. J., French, J. M., Sircus, W. 1960. *Lancet* 1:1045–48
41. Grossman, M. I., Tracy, H. J., Gregory, R. A. 1961. *Gastroenterology* 41:87–91
42. Gregory, R. A., Grossman, M. I., Tracy, H. J., Bentley, P. H. 1967. *Lancet* 2:543–44
43. McGuigan, J. E., Trudeau, W. L. 1968. *N. Engl. J. Med.* 278:1308–13
44. Gregory, R. A., Tracy, H. J., Agarwal, K. L., Grossman, M. I. 1969. *Gut* 10:603–8
45. Ellison, E. H., Wilson, S. D. 1964. *Ann. Surg.* 160:512–28
46. Aoyagi, T., Summerskill, W. H. J. 1966. *Arch. Intern. Med.* 117:667–72
47. Marks, I. N., Selzer, G., Louw, J. H., Bank, S. 1961. *Gastroenterology* 41:77–86
48. Kaye, M. D., Rhodes, J., Beck, P. 1970. *Gastroenterology* 58:476–81
49. Bonfils, S., Bader, J. P. 1970. *Progr. Gastroenterol.* 2:332–55
50. Way, L., Goldman, L., Dumphy, J. E. 1968. *Am. J. Surg.* 116:293–304
51. Cathcart, R. S., III, Webb, C. M., Otherson, H. B. Jr. 1969. *Surgery* 66: 401–4
52. Ellison, E. H., Wilson, S. D. 1967. *Progr. Clin. Cancer* 3:225–44
53. Donaldson, R. M. Jr., vom Eigen, P. R., Dwight, R. W. 1957. *N. Engl. J. Med.* 257:965–70
54. Mosahl, M. G., Broitman, S. A., Zamcheck, N. 1970. *Am. J. Clin. Nutr.* 23:336–42
55. Cook, H. B., French, A. B. 1968. *Am. J. Dig. Dis.* 13:191–203
56. Shafer, W. H. 1964. *Ann. Intern. Med.* 61:539–43
57. Wright, H. K., Kabemba, J., Herskovic, T. 1968. *Surg. Forum* 19:282–83
58. Wright, H. K., Hersh, T., Floch, M. H. 1970. *Am. J. Surg.* 119:250–53
59. Shimoda, S. S., Saunders, D. R., Rubin, C. E. 1968. *Gastroenterology* 55: 705–23
60. Mansbach, C. M., Wilkins, R. M., Dobbins, W. O., Tyor, M. P. 1968. *Arch. Intern. Med.* 121:487–98
61. Go, V. L. W., Poley, J. R., Hofmann, A. F., Summerskill, W. H. J. 1970. *Gastroenterology* 58:638–46
62. Shum, H. Y., O'Neill, B. J., Streeter, A. M. 1971. *Lancet* 1:1303

63. Friesen, S. R. 1972. *Current Problems in Surgery,* 3–52. Chicago: Yearb. Med.
64. Ballard, H. S., Frame, B. Hartsock, R. J. 1964. *Medicine* 43:481–516
65. Law, D. H., Liddle, G. W., Scott, H. W. Jr., Tauber, S. D. 1965. *N. Engl. J. Med.* 273:292–96
66. Shieber, W. 1963. *Surgery* 54:448–50
67. Murray-Lyon, I. M. et al 1968. *Lancet* 2:895–98
68. Lomsky, R., Langr, F., Vortel, V. 1969. *Nature* 223:618–19
69. Greider, M. H., McGuigan, J. E. 1971. *Diabetes* 20:389–96
70. Royston, C. M. S., Brew, D. St. J., Garnham, J. R., Stagg, B. H., Polak, J. 1972. *Gut* 13:638–42
71. Polak, J. M., Stagg, B., Pearse, A. G. E. 1972. *Gut* 13:501–12
72. Friesen, S. R., Bolinger, R. E., Pearse, A. G. E., McGuigan, J. E. 1970. *Ann. Surg.* 172:504–21
73. Dotevall, G., Westling, H. 1963. *Acta Med. Scand.* 174:777–80
74. Roberts, A., Phillips, J. P., Nezaims, J. E. 1966. *Am J. Dig. Dis.* 11: 546–52
75. Creutzfeldt, W., Arnold, R., Creutzfeldt, C., Feurle, G., Ketterer, H. 1971. *Eur. J. Clin. Invest.* 1:461–79
76. Greider, M. H., Rosai, J., McGuigan, J. E. 1974. *Cancer.* In press
77. Herbst, C. A., McGuigan, J. E. 1973. *Gastroenterology* 64:743
78. Walsh, J. H., Debas, H. T., Grossman, M. I. 1973. *Gastroenterology* 64:873
79. Trudeau, W. L., McGuigan, J. E. 1969. *N. Engl. J. Med.* 281:862–66
80. Passaro, E. Jr., Basso, N., Walsh, J. H. 1972. *Surgery* 72:60–67
81. Reeder, D. D., Jackson, B. M., Ban, J., Clendinen, B. G., Davidson, W. G. 1970. *Ann. Surg.* 172:540–46
82. Cooper, C. W., Schwesinger, W. H., Mahgoub, A. M., Ontjes, D. A. 1971. *Science* 172:1238–40
83. Care, A. D. et al 1971. *Endocrinology* 89:262–71
84. Cooper, C. W., Schwesinger, W. H., Ontjes, D. A., Mahgoub, A. M., Munson, P. L. 1972. *Endocrinology* 91:1079–89
85. Sizemore, G. W. et al 1973. *N. Engl. J. Med.* 288:641–44
86. Isenberg, J. I., Walsh, J. H., Passaro, E. Jr., Moore, E. W., Grossman, M. I. 1972. *Gastroenterology* 62:626–31
87. Bradley, E. L., Galambos, J. T., Lobley, C. R., Chan. Y. K. 1973. *Surgery* 73:550–56
88. Thompson, J. C., Bunchman, H. H., Reeder, D. D., Becker, H. D., Brandt, E. N. 1972. *Ann. Surg.* 176:384–93
89. Verner, J. V., Morrison, J. B. 1958. *Am. J. Med.* 25:374–80
90. Murray, J. S., Paton, R. R., Pope, C. E. II. 1961. *N. Engl. J. Med.* 264:436–39
91. Stoker, D. J., Wynn, V. 1970. *Gut* 11:911–20
92. Semb, L. S., Gjone, E., Rosenthal, W. S. 1970. *Scand. J. Gastroenterol.* 5:409–14
93. Zollinger, R. M. et al 1968 *Ann. Surg.* 168:502–21
94. Pierce, N. F., Carpenter, C. C. J., Elliott, H. L., Greenough, W. B. 1971. *Gastroenterology* 60:22–32
95. Barbezat, G. O., Grossman, M. I. 1971. *Lancet* 1:1025–27
96. Elias, E. et al 1972. *Lancet* 2:791–93
97. Bloom, S. R., Polak, J. M., Pearse, A. G. E. 1973. *Lancet* 1:14–16
98. Friesen, S. R. 1967. *Surgery* 62:609–13
99. Kay, A. W. 1953. *Brit. Med. J.* 2:77–80
100. Dragstedt, L. R. 1969. *Am. J. Surg.* 117:143–56
101. Berson, S. A., Yalow, R. S. 1971. *N. Engl. J. Med.* 284:445–46
102. Reeder, D. D., Jackson, B. M., Ban, J. L., Davidson, W. D., Thompson, J. C. 1970. *Surg. Forum* 21:290–91
103. Stern, D. H., Walsh, J. H. 1973. *Gastroenterology* 64:363–69
104. Isenberg, J. I., Walsh, J. H., Best, W. R., Grossman, M. I. 1972. *Gastroenterology* 62:764
105. Emas, S., Borg, I., Fyro, B. 1971. *Scand. J. Gastroenterol.* 6:39–43
106. Pearse, A. G. E., Bussolati, G. 1970. *Gut* 11:646–48
107. Kirsner, J. B., Ford, H. 1955. *J. Lab. Clin. Med.* 46:307–11
108. Hansky, J., Soveny, C., Korman, M. G. 1972. *Gastroenterology* 63:387–91
109. Stadil, F. 1972. *Scand. J. Gastroenterol.* 7:225–31
110. Berson, S. A., Yalow, R. S. 1971. *Gastroenterology* 60:215–22
111. Cope, O. 1960. *Am. J. Surg.* 99:394–403
112. Ostrow, J. D., Blanshard, G., Gray, S. J. 1960. *Am. J. Med.* 29:769–79
113. Wilder, W. T., Frame, B., Haubrich, W. S. 1961. *Ann. Intern. Med.* 58:885–93
114. Donegan, W. L., Spiro, H. M. 1960. *Gastroenterology* 38:750–59
115. Barreras, R. L., Greenlee, H. B., McConnell, C. 1969. *Clin. Res.* 17:524
116. Marx, S. J. et al 1973. *Ann. Intern. Med.* 78:371–77
117. Dent, R. I. et al 1972. *Ann. Surg.* 176:360–69

118. McGuigan, J. E., Colwell, J. A., Franklin, J. 1974. *Gastroenterology.* In press
119. Hayes, J. R., Ardill, J., Kennedy, T. L., Shanks, R. G., Buchanan, K. D. 1972. *Lancet* 1:819–21
120. Harrison, R. C., Stoller, J. L. 1971. *Am. J. Surg.* 122:198–203
121. Korman, M. G., Scott, D. F., Hansky, J., Wilson, H. 1972. *Aust. N.Z. J. Med.* 2:266–71
122. Glass, J. D., Schwartz, I. L., Walter, R. 1969. *Proc. Nat. Acad. Sci. USA* 63:1426–30
123. Clendinnen, B. G., Davidson, W. D., Lemmi, C. A. E., Jackson, B. M., Thompson, J. C. 1970. *Gastroenterology* 58:935
124. Becker, H. D., Reeder, D. D., Thompson, J. C. *Gastroenterology.* In press
125. Korman, M. G., Laver, M. C., Hansky, J. 1972. *Brit. Med. J.* 1:209–10
126. Dent, R. I., Hirsch, H., James, J. H., Fisher, J. E. 1972. *Surg. Forum* 23:312–13

Copyright 1974. All rights reserved

ANTIVIRAL CHEMOTHERAPY ❖7110

James P. Luby, M.D., M. Tracy Johnson, M.D.,[1] *and Stephen R. Jones, M.D.*

Department of Internal Medicine, University of Texas, Southwestern Medical School, Dallas, Texas

INTRODUCTION

Rather than be inclusive, this review will concentrate on antiviral chemotherapeutic agents that now have potential clinical application (amantadine, idoxuridine, cytosine arabinoside, adenine arabinoside, and methisazone). We will also briefly consider problem areas in present attempts to adapt the interferon system so that this potent, naturally occurring, broad-spectrum antiviral protein can be utilized practically in therapy.

A few general comments are in order to set the background against which the clinical evaluation of an antiviral drug may be assessed (1–4). It is apparent that although viral replication may be inhibited by the drugs under discussion at the dosages utilized, a complete in vivo "virucidal" effect probably is not presently approachable. For cure, some element of host defense mechanisms must be intact. The pre-existing level of immunity is also important. For example, primary varicella in a renal transplant recipient may intrinsically be a much more difficult disease state to treat than herpes zoster in a normal host, even though equal drug concentrations in serum and tissue are attained. The therapeutic response should be gauged in relationship to the stage of disease at which treatment is begun. Irreversible damage occurs rapidly in herpes simplex encephalitis and a patient in the third week of this disease should be expected to have a less favorable outcome than one treated earlier. Consideration should also be focused on elements of the pathologic process that may be independent of viral replication such as delayed hypersensitivity responses to antigenic components. In this regard, the stromal phase of herpes simplex keratitis and any attendant iritis can be cited.

The toxicity of the drug must be measured. In the future, this will extend to possible interactions with other drugs, since patients most at risk with respect to the serious consequences of DNA viral infections often are receiving immunosuppressive therapy which is in itself toxic. To what extent is the leucopenia induced by chronic azathioprine therapy influenced by the addition of adenine arabinoside, a drug presently thought to have little if any hematological toxicity? Ideally in the clinical evaluation of any antimicrobial compound, the agent's level of sensitivity to the drug as assessed by in vitro tests should be able to be related to concentrations actually attained in serum and tissue. In the field of antiviral chemotherapy, however, variables remain to be standardized before in vitro sensitivity measurements can be applied exactly to the clinical situation. These include the type of test (zone of inhibition around a standardized disc, cytopathic effect inhibition, plaque reduction), cell system used, effect of passage history of the isolate, the constituents of the nutrient media, and its pH (5–10). That such tests even at the present stage of development may be important in the evaluation of an antiviral drug is supported by the following considerations: (*a*) it is unproven that all isolates of a given virus are uniformly susceptible to the same drug concentration. Lack of clinical response may be explained by inherent resistance of an isolate to the drug; and (*b*) resistant mutants may emerge and influence the clinical outcome.

---

[1] Major, MC, USAF, Air Force Institute of Technology.

After relative safety has been established, the essential task in evaluating an antiviral drug is to prove clinical efficacy. This necessitates random double-blind controlled studies. Laboratory documentation of infection should be rigorous and the patients must be shown to have been carefully matched. Although serving a purpose, individual case reports or reports of series of patients treated without controls cannot be relied upon to give the medical community the answers that it needs to guide therapy in the future.

AMANTADINE

Amantadine hydrochloride (*l*-adamantanamine hydrochloride, aminoadamantane hydrochloride, Symmetrel®) is the synthetic salt of a symmetric C-10 primary amine. Discovered during the course of empiric screening procedures, present clinical interest focuses on its effect against influenza A viruses (11). The name was derived from the word "adamant," meaning firm or unyielding, and refers to the stability of the compound.

Antiviral Spectrum

In tissue culture, influenza A and C viruses are inhibited by amantadine. There was no effect against influenza B virus (11–15). Influenza A2 strains appeared most sensitive, but within this group a certain variability was apparent. For example, A2/Scotland/49/57 was shown to have a greater sensitivity than A2/Sing/1/57 (12). The presently circulating A2 strains (Hong Kong) have generally been shown to be relatively sensitive to amantadine. Studies in eggs and experimental infections in mice and ferrets have confirmed the susceptibility of influenza A viruses to amantadine.

Recent in vitro susceptibility studies have shown that the replication of all arenaviruses tested to date have been reduced in the presence of amantadine (16,17). The effect is present for the prototype of this group of viruses, lymphocytic choriomeningitis virus, and extends to Junin virus, the etiologic agent of Bolivian hemorrhagic fever. It is not known if Lassa fever virus, another arenavirus, is affected and no clinical studies have been performed. An in vitro effect on rubella virus has been documented but subsequent studies revealed a complete lack of efficacy of amantadine in the prophylaxis of naturally occurring rubella infections (18).

Mechanism of Action

Amantadine is thought to exert its effect on influenza A virus at an early step in viral multiplication. The step most likely to be affected is virus penetration into the host cell (19–22). The evidence favoring this mechanism of action can be summarized as follows: (*a*) No inactivation occurs when amantadine is added directly to suspensions of virus. (*b*) There is no interference with viral adsorption to the host cell surface. (*c*) In the presence of amantadine, adsorbed infective virus is retained at the cell surface. This virus is susceptible to inactivation by antibody. (*d*) Amantadine is effective in inhibiting single growth cycles of virus only when added prior to infection or immediately thereafter. (*e*) There is no effect on viral neuraminidase. (*f*) Early nuclear and cytoplasmic viral antigens do not appear during treatment with amantadine. In the case of the arenaviruses, amantadine acts not only to interfere with virus penetration but also affects late viral synthesis and release of progeny virus from the cell (16).

Pharmacology

Amantadine is resistant to biological degradation and there is no evidence for metabolism of the drug in man (23). It is well absorbed from the gastrointestinal tract and excreted primarily in the urine. In the mouse, concentration of the drug occurs in lung tissue with heart muscle concentrations being intermediate between lung and blood.

Clinical Studies

This article will concentrate on the effect of amantadine in situations where natural challenge with influenza A2 virus has occurred. Artificial challenge experiments in volunteers have been presented and reviewed (24, 25). Two types of studies will be described: prophylactic and therapeutic.

PROPHYLAXIS The results of five representative double-blind studies with adequate laboratory documentation of infection assessing the role of amantadine against influenza A2 virus in man under natural challenge conditions can be summarized as follows: (*a*) Amantadine exerted a significant prophylactic effect in preventing clinical disease due to influenza A2 virus. The overall level of protective efficacy in these five studies was 66% (all patients combined disregarding pre-existing antibody levels). (*b*) A lesser inconstant effect was present suggesting that under some circumstances amantadine might prevent influenza A2 virus infection, as determined by a significant rise in antibody titer. Antibody titers in convalescent serum specimens also tended to be lower in persons given amantadine. (*c*) The effect of amantadine in the prevention of clinical disease may have been more pronounced if a significant level of pre-existing antibody was present (26–30). Only one of the five studies found no effect on the prevention of clinical illness and these results have been related to the absence of pre-existing antibody in most of the participants (28). In one of the studies it was found that the clinical illness that resulted tended to be milder in persons receiving amantadine. In that study, mild, moderate, and severe clinical illnesses were found in 56, 35, and 9%, respectively, of 200 persons who developed serologically documented illness while on amantadine. These same figures were 38, 43, and 19%, respectively, for the control group (30).

THERAPY Amantadine has been shown to have a certain therapeutic effect if given early ($\leq$ 20 hr after onset) to persons with clinical illness due to influenza A2 virus (31, 32). This effect was shown with prison inmates in Texas and Maryland where patients were seen early in the course of their disease and where frequent observations could be made by a limited number of study personnel. Persons with a rapid resolution of their illnesses (temperatures less than 100°F in 24 hr and 50% or more clearing of symptoms within the first 36 hr after onset) were significantly more frequent in the amantadine-treated group than in the control population. In this study, the virus was recovered at high rate in both treatment and placebo groups, indicating no effect on virus shedding. Systemic as well as local antibody increases that developed in amantadine-treated patients during convalescence were equivalent to those of placebo-treated patients. Since amantadine has no known antipyretic or analgesic properties, its efficacy seemed best related to inhibition of virus replication.

In contrast, in a study conducted in Great Britain with patients who were seen by 57 family doctors, there was a reduction from 75 to 47 hr ($p < 0.01$) in mean duration of fever in treated individuals compared with those receiving placebo, but no significant effects were observed on other influenzal symptoms (33). The lack of effect on these other symptoms was possibly explained, in part, by circumstances that permitted less intense observation and by the multiplicity of persons involved in gauging the symptoms. There was no evidence that amantadine treatment significantly depressed the levels of circulating influenza antibodies detected in convalescent sera.

In another study, although a therapeutic effect seemed clear, it was stated that "a striking amelioration of clinical findings does not result from treatment" (34). Influenza A2 (Hong Kong) virus excretion measurements were also performed in this study and were significantly different only at or before 10 hr of therapy. The viral excretion studies in persons developing natural influenza explain the inconsistent effects related to secondary case attack rates in close contacts. One study has suggested that amantadine therapy might make the person less infectious, but no effect on secondary case attack rates has been reported (33). The effect of amantadine therapy on the rate of bacterial superinfections after influenza has not yet been assessed.

Toxicity

Side effects include increased jitteriness, inability to concentrate, insomnia, depression, tremors, confusion, hallucinations, and occasionally drowsiness. The incidence is dose- and probably age-related (elderly persons being more susceptible), but, in general, at a low level. Some studies have reported no adverse side reactions. Withdrawal of the drug results in disappearance of the side effects. Recently, improvement in Parkinson's disease has been reported after therapy with amantadine (35). An interaction with central dopamine metabolism has been postulated and it seems possible that such an effect might explain, in part, the etiology of some of the side reactions encountered in influenza prophylaxis. Livedo reticularis is a common side effect of treatment with amantadine for Parkinson's disease and this may be due to depletion of catecholamine stores in peripheral nerve terminals (36). Convulsions have occurred in a patient receiving 800 mg amantadine a day. Potentiation of insulin effect has been reported in a single patient with previously stable diabetes mellitus. The potential teratogenicity of amantadine has not been excluded and appropriate caution should be exercised in prescribing this drug for pregnant women.

Dose

The daily adult dose is 100 mg orally b.i.d. In children one to nine years of age, 2–4 mg/lb b.i.d. should be given.

Conclusions

Although not the ideal antiviral drug, situations occur where the administration of amantadine may be indicated (37, 38). If influenza A virus is documented as circulating in the community and the level of immunity to this strain in the population is low, amantadine might be considered for use as a prophylactic agent in, for example, household contacts of clinical cases, hospital emergency room personnel, and to prevent nosocomial influenza infection (39). The cost and potential side effects of amantadine as opposed to the use of a simple analgesic and antipyretic like aspirin should be considered before applying this drug therapeutically. Future studies remain to show whether amantadine administration as therapy may be associated with a lesser incidence of influenzal complications.

IDOXURIDINE

Originally synthesized for use in tumor chemotherapy, idoxuridine (5-iodo-2'-deoxyuridine, IDUR, IDU) soon became recognized as an antiviral agent.

Antiviral Spectrum

In tissue culture, idoxuridine has been shown to be effective against DNA-containing viruses. In particular, an in vitro effect has been demonstrated against herpes simplex virus (types 1 and 2), varicella-zoster virus, cytomegalovirus, and vaccinia.

Mechanism of Action

In cells, idoxuridine is successively converted to its mono-, di-, and triphosphate. It acts as a thymidine analogue and is incorporated into both host cell and viral DNA in place of thymidine. Electron microscopy of herpesvirus-infected cells after idoxuridine addition reveals large numbers of defective particles. Partially assembled capsomeres, filamentous material, and ragged, irregularly sized particles without envelopes result (40). The best explanation for these findings is that the DNA, resulting after idoxuridine addition, is "fraudulent" and cannot code for processes related to assembly or for synthesis of structural proteins. Host cell DNA is also affected. In the intact organism, rapidly proliferating tissues (bone marrow, gastrointestinal tract, hair) are especially involved (41). In tissue culture, the emergence of idoxuridine-resistant mutants occurs relatively commonly (42).

Pharmacology

In man, idoxuridine is both rapidly excreted and metabolized (41). Four hours after completion of a 2-hr infusion of ^{131}I-labeled idoxuridine in two volunteers with terminal cancer, 44% of injected radioactivity had been collected in the urine; after 24 hr, approximately 90% had been excreted. During the 2-hr period of infusion, half the radioactivity excreted was in the form of idoxuridine. After this period, progressively more radioactivity was found in metabolic products (5-iodouracil and iodide). Idoxuridine is not significantly bound to serum proteins and has been shown to be present in cerebrospinal fluid.

Clinical Studies

Kaufman (43) pioneered the use of idoxuridine in the treatment of herpes simplex keratitis. Later clinical attention has concentrated on idoxuridine as therapy for herpes simplex encephalitis and generalized infections due to this virus.

HERPES SIMPLEX KERATITIS A common cause of blindness, herpetic keratitis can be classified by stage of involvement [epithelial, stromal (superficial or deep), and kerato-iritis]. The virus multiplies primarily in the epithelial cells of the cornea producing the characteristic dendritic ulcer. Different strains probably vary in their capacity to invade the stroma. In the usual case, virus is isolated with difficulty from the stroma. The stromal reaction and the iritis seemed caused primarily from delayed hypersensitivity responses to viral antigens (44). Secretory IgA appears in tears and the natural history of the disease in man lasts one to four weeks. Relapses are common and possibly related to the continuing presence of virus in such structures as the lachrymal gland.

When compared to placebo, idoxuridine therapy results in a more rapid decrease in viral titer. If instituted early in the course of the disease process, idoxuridine has proven therapeutic efficacy in double-blind studies with placebo controls. Therapeutic success rates with idoxuridine ranged from 47–92% in seven studies (45). An overall success of approximately 71% (119/168) can be estimated from these seven studies as opposed to 24% (23/95) for control groups. Idoxuridine therapy probably has no effect on recurrence rates. Resistant isolates have been reported from patients who were treatment failures (45).

HERPES SIMPLEX ENCEPHALITIS Herpes simplex virus is the most common known cause of sporadic severe viral encephalitis. Although herpes simplex virus can produce aseptic meningitis, the more common clinical presentation is that of an asymmetric, often lethal encephalitic process. Sequelae are common in persons who survive the encephalitic illness. As in herpetic keratitis, herpesvirus type-1 isolates are almost always recovered from children (non-neonates) and adults with encephalitis.

Double-blind studies have not been performed assessing the efficacy of idoxuridine in the treatment of herpetic encephalitis. In the absence of these controlled studies, proof that idoxuridine influences the natural history of this disease comes largely from the compilation of cases that have or have not been treated with this drug. In evaluating these kinds of data using historical controls, the following considerations should be kept in mind: (*a*) With greater awareness of the disease, less severe cases may now be diagnosed. (*b*) The practice of performing brain biopsy for diagnostic purposes with a craniotomy and neurosurgical decompression procedure may, in itself, be therapeutic. (*c*) Other adjunctive supportive measures may have improved. In recent literature reviews, the overall case-fatality ratio for herpes simplex encephalitis without drug therapy has been determined to be 55% (60/110) (46, 47). If only cases with coma during their course are considered, the case-fatality ratio rises to 71% (41/58). With idoxuridine treatment, the overall case-fatality ratio is 47% (15/32), a figure not significantly different from 55%. However, only 40% (7/19) of comatose patients died. The difference between 71% and 40% is statistically significant ($p < 0.01$). Sequelae (severe deficits) could be adequately evaluated in lesser numbers of patients and were not significantly different between the untreated group, 68% (13/19), and the idoxuridine-treated group, 56% (9/16).

The largest series of reported cases of herpes simplex encephalitis treated with idoxuridine stems from Michigan (48, 49). Between 1966 and 1971, 20 patients were treated; 18 patients survived for a full five-day course of therapy. Six of these patients later died, with at least three dying from toxicity directly related to the drug. Of the 12 survivors, 9 were considered to have recovered without sequelae. This figure was contrasted with 1 of 6 survivors recovering without sequelae in a total group of 9 patients diagnosed in Michigan during the same interval but not treated with idoxuridine.

GENERALIZED INFECTION IN THE NEONATE Type-2 herpesvirus is the etiologic agent in this disorder. In tissue culture, these isolates generally are more resistant to idoxuridine. Of 7 neonates reported by different authors as treated with idoxuridine, only 1 could be considered normal following recovery from infection; 2 died during the immediate illness, and another died one month later from pneumonia. Three have significant residual abnormalities (46).

OTHER INFECTIONS WITH HERPES SIMPLEX VIRUS Topical idoxuridine has been found to have no effect in the prevention or therapy of herpes labialis (50).

VARICELLA-ZOSTER INFECTIONS Topical application of a 40% idoxuridine solution in dimethylsulfoxide has been reported to be therapeutic in the treatment of herpes zoster (51). Dimethylsulfoxide is not licensed for use in the United States.

CYTOMEGALOVIRUS Although a suggestion of therapeutic efficacy was noted in one infant with congenital cytomegalic inclusion disease, there has been no consistent effect (52).

Toxicity

Side effects after intravenous administration include leucopenia, thrombocytopenia, stomatitis, alopecia, fingernail loss, and occasionally jaundice. Toxicity is dose related. In the Michigan series, the mean time in days to the time of maximal thrombocyte depression after the cessation of treatment was five days. To be noted in this series was the occurrence of hemorrhage at the site of tracheostomy, a complication related to the thrombocytopenia and causing death from respiratory insufficiency in 3 patients (49).

Dose

Most of the clinical experience with idoxuridine has been with the slow infusion of this drug over a 24-hr period. However, the Michigan workers, on the basis of measurements of antiviral activity in serum, now recommend infusion of the drug over a 45–60 min interval every 12 hr for 5 days. The daily dose is 54 mg/kg, with the total 5-day dose never exceeding 20 g (49, 53). Idoxuridine is prepared just before administration as a solution in 5% dextrose–water.

Conclusions

Idoxuridine is of accepted value topically in the therapy of herpes simplex keratitis. A certain efficacy in the treatment of herpes simplex encephalitis seems probable but remains to be incontestably demonstrated by double-blind controlled studies.

CYTOSINE ARABINOSIDE

Cytosine arabinoside (1-β-D-arabinofuranosylcytosine, cytarabine, Ara-C, Cytosar®), a compound of proven efficacy in the therapy of acute granulocytic leukemia, also has been shown to have antiviral properties.

Antiviral Spectrum

In tissue culture and experimental animals, Ara-C has been found to inhibit the replication of certain DNA viruses, including herpes simplex virus (types 1 and 2), varicella-zoster virus, cytomegalovirus, and vaccinia (54). The order of sensitivity of human DNA viruses to Ara-C indicates that varicella-zoster is probably most sensitive, followed by type-1 herpes simplex virus, vaccinia, and lastly cytomegalovirus. A strain of varicella-zoster virus has been shown to be sensitive to 0.1 μg/ml Ara-C (90% inhibition), whereas significant reduction of cytomegalovirus yield begins between 1 and 10 μg/ml, and is not complete at 100 μg/ml (55, 56).

Mechanism of Action

In experiments with clones of cells resistant to the action of Ara-C, it was found that a phosphorylated derivative is the effective inhibitor. Unlike idoxuridine, Ara-C is incorporated into viral and/or host cell DNA only to a limited extent. It appears probable that the mechanism of action of Ara-C resides in its inhibition of enzyme systems responsible for the production of essential DNA components. In particular, the formation of deoxycytidine may be affected as demonstrated by experiments in which the addition of this compound competitively reverses the action of Ara-C. The activity of DNA polymerase has also been shown to be affected by Ara-C. In contrast to idoxuridine, the emergence of type-1 herpesviruses resistant to Ara-C may be limited. Those strains of herpes simplex virus resistant to idoxuridine have, to date, always been sensitive to Ara-C.

Pharmacology

Ara-C in man is rapidly metabolized to an inactive compound, Ara-U (uracil arabinoside), by deaminating enzymes primarily present in the kidney and liver. After administration of Ara-C, 5–10% of the drug can be recovered in the urine unchanged; 85–95% of the dose is found in the urine in the form of Ara-U. Plasma levels of Ara-C and Ara-U have been measured by radioisotope dilution studies after single intravenous doses of Ara-C or during constant infusion (57). After a single intravenous infusion of Ara-C, plasma levels declined rapidly during the first 30 min; decay after this period was slower. Peak levels of Ara-C in plasma after the infusion of 67 mg/m^2, 200 mg/m^2, and 3000 mg/m^2 were between 0.5–1, 5–10, and 100–500 μg/ml, respectively. After a loading dose and during constant intravenous infusion of 112 mg/m^2/24 hr, a steady state level of Ara-C in plasma ranged between 0.05–1 μg/ml. These values of Ara-C in plasma can be related to the in vitro sensitivities of various DNA viruses but should be interpreted with caution for reasons previously outlined. If Ara-C is given by rapid infusion, larger doses of the drug are needed because of the rapid metabolism of the compound.

Ara-C does cross the blood-brain barrier and enter the cerebrospinal fluid (CSF). The entrance is delayed, and this probably accounts for the very low concentrations of drug found in CSF after single intravenous infusions. During constant intravenous administration, the level of Ara-C in CSF was 40% that of simultaneous plasma samples. Intrathecal therapy with Ara-C increases the CSF concentration but whether this results in increased brain tissue levels of Ara-C is unknown.

Clinical Studies

Ara-C has, to date, clinically been utilized most extensively in the therapy of varicella-zoster infections.

VARICELLA-ZOSTER Enthusiasm for the treatment of severe varicella-zoster infections stems from the report in which 6/8 children, all compromised hosts with progressive viral disease, appeared to respond to Ara-C therapy (58). Five of the children had pulmonary involvement and the two deaths were among these individuals. Spontaneous cure of such infections may be presumed to be very unlikely. Other authors have reported cases of varicella-zoster infections that also seemed to respond to Ara-C therapy (59–63). These observations have been uncontrolled and recent reports have questioned the therapeutic efficacy of Ara-C. These latter reports have included the lack of response of new lesion occurrence to Ara-C therapy in a leukemic child with primary varicella and the inability to distinguish between drug- and placebo-treated patients with herpes zoster in a double-blind study utilizing 150 mg/m^2 of Ara-C administered over a 36-hr period (64, 65). Further double-blind studies are now in progress. In efforts to circumvent the hematological toxicity and possible immunosuppressive effects of Ara-C at full dosage levels (100 mg/m^2/day for 4–5 days), smaller amounts of drug have been given (10-100 mg/m^2/day for 1.5–7 days) (66). The initial studies were reported as promising but again should be interpreted with caution as controls were not included.

HERPES SIMPLEX Individual case reports and series have appeared indicating cure of herpetic encephalitis, eczema herpeticum, and severe gingivostomatitis in the compromised host (47, 62, 66, 67). These studies were uncontrolled.

CYTOMEGALOVIRUS Urinary excretion of cytomegalovirus in congenital infection represents a readily measured objective response to the institution of antiviral chemotherapy. With doses of Ara-C sufficient to induce thrombocytopenia, only a transient drop in viral excretion was recorded in four infants. There was no associated clinical response (56). Similar results have been recorded (68). Although another report indicated that there might be some therapeutic efficacy, Ara-C does not seem to be a promising agent for the treatment of congenital disease due to cytomegalovirus (69).

Toxicity

Hematological changes are of primary importance (70). Anemia, reticulocytopenia, and megaloblastoid changes occur. Leucopenia, related to a decrease in the number of circulating granulocytes, and thrombocytopenia may supervene. The drug interferes with the primary immune response in experimental animals (71). Anorexia, nausea, and vomiting are the chief manifestations of gastrointestinal toxicity. Mucosal ulceration is rare. Chromosomal changes have been reported acutely and Ara-C is teratogenic in experimental animals (rats, chicks) (72, 73). Hepatic toxicity is probable at certain dosages (56). Phlebitis at the intravenous infusion site may occur. Corneal speckling may complicate topical therapy of herpetic keratitis.

Dose

The majority of clinical experience is with 100 mg/m^2/day as a constant intravenous infusion for three to five days.

Conclusions

The rational use of Ara-C in most of the disease states under consideration awaits double-blind confirmation.

ADENINE ARABINOSIDE

Adenine arabinoside (arabinofuranosyladenine, Ara–A, Vidarabine®) was originally noted to have antiviral properties by two separate groups of investigators (74, 75).

Antiviral Spectrum

Antiviral activity of Ara-A in tissue culture and experimental animals extends to the following DNA viruses: herpes simplex virus, types 1 and 2, varicella-zoster virus, cytomegalovirus, and vaccinia (74–78). Variable activity against adenovirus type 3 has also been demonstrated. Of theoretical interest is the effect of Ara-A against Rous sarcoma virus, an RNA tumor virus dependent on DNA synthesis. In tissue culture, some strains of herpes simplex virus type 1 were inhibited $\geq 99\%$ at drug concentrations of 10 μg/ml (77). Significant reduction of varicella-zoster virus yield ($\geq$ 2 logs) in human embryonic lung diploid fibroblasts began at 2.5 μg/ml, with all strains tested to date being sensitive at 20 μg/ml. Herpes simplex virus type 2 and cytomegalovirus tend to be more resistant in these in vitro tests. The principal metabolite of Ara-A, hypoxanthine arabinoside (Ara-Hx), also possesses antiviral properties which, however, on a weight basis, are less than those of Ara-A. Only one of seven varicella-zoster virus strains showed $\geq 2 \log_{10}$ reduction in virus yield in human embryonic lung cells at 20 μg/ml (79).

Mechanism of Action

The mechanisms of antiviral action of Ara-A are incompletely understood (80–85). The active compounds affecting viral inhibition are thought to be the phosphorylated derivatives of Ara-A. Three possible mechanisms have been postulated: (*a*) inhibition of DNA polymerase, (*b*) inhibition of ribonucleoside reductase, and (*c*) incorporation of the monophosphorylated derivative of Ara-A into DNA with subsequent chain termination. Present evidence indicates that the first two possible mechanisms are more likely to be operative significantly in human cells but further work needs to be done on this point. The development of resistance to Ara-A has not been observed after repeated exposures of herpes simplex virus type 1 to drug either in Hep-2 cells or in a mouse encephalitis model (77, 86).

Pharmacology

Ara-A is a white crystalline powder with limited solubility in water. It is poorly and erratically absorbed when given orally, and hence must be given parenterally. After parenteral administration, Ara-A is rapidly deaminated to Ara-Hx. The major pathway of excretion of Ara-A and Ara-Hx is into the urine. In man, 60–70% of tritium-labeled drug expressed as nonvolatile tritium can be recovered in the urine (87).

Only limited studies of drug levels in humans given therapeutic doses of Ara-A have been performed. To determine valid drug concentrations, an inhibitor of the enzyme deaminating Ara-A to Ara-Hx must be added to the specimen immediately after collection. Intravenous administration of 10 mg/kg as a 6-hr infusion has resulted in plasma levels of Ara-A from 1–6 μg/ml and Ara-Hx of 1–7 μg/ml. Within one hour of the end of infusion, only Ara-Hx was detectable. Accumulation of Ara-Hx in plasma was noted in one patient with compromised renal function given full therapeutic doses of the drug. The drug does penetrate into the cerebrospinal fluid, but the relative proportion of Ara-A to Ara-Hx has not been determined (79). In equilibrium dialysis experiments, Ara-A does not appear to be significantly protein-bound; no data are available for Ara-Hx.

Clinical Studies

Completed controlled clinical trials evaluating Ara-A as a therapeutic agent have been limited to the therapy of herpes simplex keratitis (88). Results indicated that when topically applied, Ara-A was equivalent to idoxuridine in the treatment of superficial epithelial disease, and when combined with topical steroids may have been superior when there was deep active stromal involvement and iritis. Several patients who had been refractory to idoxuridine were controlled with Ara-A ointment.

Preliminary studies assessing Ara-A as a systemic antiviral agent have been performed, particularly at the University of Alabama (79, 89). Encouraging results have been found particularly with progressive mucocutaneous disease due to herpes simplex virus type 1 and herpes zoster in compromised patients where some element of host defense mechanisms continued intact. Less encouraging results were observed in compromised hosts with extending genital and anorectal ulcerations due to herpes simplex virus type 2. Infants with congenital cytomegalic inclusion had a transient diminished viruria with Ara-A but their course was not otherwise altered. None of these studies have shown unequivocal efficacy of parenteral Ara-A in humans, but double-blind trials are now in progress for encephalitis, generalized disease in neonates, and mucocutaneous disease due to herpes simplex virus, and in herpes zoster.

Toxicity

During parenteral administration of Ara-A, nausea and vomiting occurs in approximately 25% of patients, and has been easily controlled by antiemetics. Hematological toxicity (leucopenia, drop in hemoglobin values) has been encountered only infrequently and could not unequivocally be related to the drug itself. Chromosome gaps and open breaks have been demonstrated in human leucocyte cultures treated with Ara-A and teratogenicity has been observed in experimental animals treated during gestation (90, 91). Ara-A does not appear to be immunosuppressive in experimental animals (86).

Dose

Current experience with parenteral Ara-A is with 10–15 mg/kg/day given daily as an intravenous infusion over a 6- to 12-hr period. The presently available form of the drug is soluble only to the extent of 0.5 mg/ml, necessitating at least a liter of fluid daily in most adults.

Conclusions

Ara-A is a promising new drug against DNA viruses, which apparently shows only minor toxicity in humans, although more information is needed in this regard. In experimental animals, it does not affect the immune response. It would appear to be as effective as idoxuridine in herpes simplex keratitis. Controlled studies, now in progress, should be able to determine its efficacy in disease states caused by herpes simplex and varicella-zoster viruses.

METHISAZONE

Methisazone (N-methylisatin β-thiosemicarbazone, Marboran®) has been shown in tissue culture and experimental animals to exert an effect on variola and vaccinia viruses (92, 93). The compound must be present at the initiation of infection and exerts its action by interfering with the translation of late messenger viral RNA. As a result, certain structural proteins are not produced and a mature, fully infectious virion does not eventuate.

Methisazone has been used in the prophylaxis of smallpox. It has no therapeutic benefit in the treatment of established clinical variola. In the United States, it has been used in the therapy of vaccination reactions, particularly progressive vaccinia. With the end of compulsory vaccination in this country, it can be expected that the role of methisazone as a therapeutic agent will be diminishing.

In four separate trials (Madras, India, 1963; Madras, India, 1965–1967; Brazil, 1964–1965; West Pakistan, 1964–1970), methisazone was shown to have a prophylactic effect against variola (94–97). The level of overall protection by respective study as indicated above was 83, 71, 74, and 47%. The administration of methisazone concurrently with vaccination against smallpox probably results in a reduction of successful "takes" in primary vaccinees and a more pronounced reduction in revaccination "take" rates. Serologic studies (fourfold rises in antibody titer), however, indicate that vaccination is equally successful in raising antibody titer levels in drug and control groups (primary vaccinees and revaccinees). The conclusions to be drawn from these studies are that the primary method to reduce smallpox occurrence in contacts of cases and in aborting epidemics must be vaccination with methisazone playing an adjunctive role (97).

Methisazone is indicated in progressive vaccinia along with vaccinia immune globulin. Without these measures, this complication of vaccination, usually occurring in the compromised host, is invariably fatal. Methisazone is also indicated in eczema vaccinatum and generalized vaccinia when these disorders cannot be controlled by vaccinia immune globulin (98). Nausea and vomiting are common side effects. Chemical tests to detect early hepatic toxicity should also be performed.

INTERFERON

Since its discovery in 1957 by Isaacs & Lindenmann, interferon has been excitedly looked to for its promise as an antiviral chemotherapeutic agent (99–104). Although much has been learned about this substance, problem areas exist—particularly about its practical utilization in the treatment of human viral diseases. There are now efforts to confront some of these problem areas directly to determine if they can be circumvented or are as formidable as might have been supposed. These efforts should culminate in greater numbers of controlled human trials of interferon and interferon inducers in the prophylaxis and therapy of viral illnesses. Hopefully, information can be gained from such trials to determine efficacy and to guide further practical development.

Problems associated with the preparation of exogenous human interferon and its administration as a prophylactic or therapeutic agent include those related to production, purification and safety, possible lability of preparations under field conditions, doses required, and the practical difficulties associated with licensure. Since its action is species specific, production of interferon necessitates the availability of large numbers of human cells. This requirement may be answered by blood and serum banks where leucocytes could be processed. Diploid human cell strains could also be used. After treatment of such cells with a viral inducer (Newcastle disease virus, Sendai virus), interferon in yields approximating 20,000 units/ml could be produced. With further development, greater quantities might be expected. Once produced, interferon must be purified and the safety of the preparation demonstrated. It must be free of adventitious viruses and this extends to hepatitis A and B viruses, cytomegalovirus, and Epstein-Barr virus, which are known to be present in blood. Loss of interferon in use under field conditions can be minimized by lyophilization or freezing. The effective dose would be measured during clinical trials but it should be remembered that the serum clearance of this substance is rapid. In established viral diseases, exogenous interferon as therapy should be able to increase concentrations already present as a result of natural infection. In varicella-zoster virus infection, large quantities of interferon are present naturally at sites of viral replication (105). Last, the preparation must be licensed and this necessitates studies determining its efficacy and short and long term safety. Although such problems are difficult, they are not impossible as evidenced by the use of blood products for other purposes and the present widespread availability of interferon preparations in the Soviet Union for topical application in, for example, the prophylaxis of influenza (106).

Interferon-inducers are also being developed for their use in the prophylaxis and therapy of human viral illnesses. With regard to practical application, problem areas exist in relationship to their toxicity, potential for antigenicity, limited duration of effect, and possible hyporesponsiveness of the host with a natural viral infection to interferon induction (107). The most extensively studied inducer is the double-stranded RNA macromolecule, polyriboinosinic acid/polyribocytidilic acid, or Poly I•C (108). Although the major action of Poly I•C is accounted for by its capacity to induce interferon, other effects occur that explain part of the benefit occurring during therapy and include: (*a*) possible direct chemotherapeutic action through inhibition of virus-induced protein synthesis, and (*b*) direct enhancement of host immunological mechanisms (antibody, delayed hypersensitivity responses, and phagocytic activity of the reticuloendothelial system). Toxicity is species-related, being particularly pronounced in the dog but much less in monkeys and in man. Its pertinent toxicity for man includes pyrogenicity (release of endogenous pyrogen), potential teratogenicity, and capacity to elicit hepatic functional abnormalities. This compound probably has a favorable therapeutic ratio when applied topically in man (eye, respiratory tract). The future promise of interferon-inducers such as Poly I•C in the practical therapy of human viral illnesses is attested to by a recent study performed by Italian workers (109). Following demonstration of the efficacy of Poly I•C in the treatment of herpetic keratitis in rabbits (110), these investigators obtained a favorable response in 81% (17/21) of patients with this disorder, while this result in matched controls was attained in only 42% (5/12) of persons treated with idoxuridine. Although only limited numbers of patients were treated, the study suggests efficacy and indicates promise in this line of approach, especially for the future when inducers with less inherent toxicity might be developed.

Literature Cited

1. Staehelin, M. 1959. *Progr. Med. Virol.* 2:1–42
2. Kaufman, H. E. 1965. *Progr. Med. Virol.* 7:117–59
3. Hirschman, S. Z. 1971. *Am. J. Med.* 51: 699–703
4. Borden, E. C., Carter, W. A. 1972. *Medicine* 51:189–90
5. Herrmann, E. C. Jr. 1961. *Progr. Med. Virol.* 3:158–92
6. Herrmann, E. C. 1961. *Proc. Soc. Exp. Biol. Med.* 107:142–45
7. Renis, H. E. 1970. *Cancer Res.* 30: 189–94
8. Walker, W. E., Waisbren, B. A., Martins, R. R., Batayias, G. E. 1971. *Am. J. Clin. Pathol.* 56:687–92
9. Fiala, M., Chow, A., Guze, L. B. 1972. *Antimicrob. Ag. Chemother.* 1:354–57
10. Jones, S. R., Luby, J. P., McElligott, D. Unpublished
11. Davies, W. L. et al 1964. *Science* 144: 862–63
12. Schild, G. C., Sutton, R.N.P. 1965. *Brit. J. Exp. Pathol.* 46:263–73
13. Tyrrell, D. A. J., Bynoe, M. L., Hoorn, B. 1965. *Brit. J. Exp. Pathol.* 46:370–75
14. Neumayer, E. M., Haff, R. F., Hoffmann, C. E. 1965. *Proc. Soc. Exp. Biol. Med.* 119:393–96
15. Oxford, J. S., Logan, I. S., Potter, C. W. 1970. *Ann. NY Acad. Sci.* 173:300–13
16. Welsh, R. M., Trowbridge, R. S., Kowalski, J. B., O'Connell, C. M., Pfau, C. J. 1971. *Virology* 45:679–86
17. Pfau, C. J., Trowbridge, R. S., Welsh, R. M., Staneck, L. D., O'Connell, C. M. 1972. *J. Gen. Virol.* 14:209–11
18. Maassab, H. F., Cochran, K. W. 1964. *Science* 145:1443–44
19. Hoffmann, C. E., Neumayer, E. M., Haff, R. F., Goldsby, R. A. 1965. *J. Bacteriol.* 90:623–28
20. Oxford, J. S., Schild, G. C. 1968. *J. Gen. Virol.* 2:377–84
21. Goedemans, W. T. 1970. *Zentralbl. Bakteriol.* 214:289–91
22. Long, W. F., Olusanya, J. 1972. *Arch. Gesamte Virusforsch.* 36:18–22
23. Bleidner, W. E., Harmon, J. B., Hewes, W. E., Lynes, T. E., Hermann, E. C. 1965. *J. Pharmacol. Exp. Ther.* 150: 484–90
24. Jackson, G. G., Muldoon, R. L., Akers, L. W. 1963. *Antimicrob. Ag. Chemother.* 3:703–7
25. Sabin, A. B. 1967. *J. Am. Med. Assoc.* 200:135–42
26. Quilligan, J. J., Hirayama, M., Baernstein, H. D. 1966. *J. Pediat.* 69:572–75
27. Galbraith, A. W., Oxford, J. S., Schild, G. C., Watson, G. I. 1969. *Lancet* 2: 1026
28. Galbraith, A. W., Oxford, J. S., Schild, G. C., Watson, G. I. 1969. *Bull. WHO* 41:677–82
29. Oker-Blom, N. et at 1970. *Brit. Med. J.* 3:676–78
30. Smorodintsev, A. A. et al 1970. *Ann. NY Acad. Sci.* 173:44–61
31. Hornick, R. B., Togo, Y., Mahler, S., Iezzoni, D. 1969. *Bull. WHO* 41: 671–76
32. Togo, Y. et al 1970. *J. Am. Med. Assoc.* 211:1149–56
33. Galbraith, A. W., Oxford, J. S., Schild, G. C., Potter, C. W., Watson, G. I. 1971. *Lancet* 2:113–15
34. Couch, R. B. 1969. *Bull. WHO* 41: 695–96

35. Schwab, R. S., England, A. C. Jr., Poskanzer, D. C., Young, R. R. 1969. *J. Am. Med. Assoc.* 208:1168–70
36. Shealy, C. N., Weeth, J. B., Mercier, D. 1970. *J. Am. Med. Assoc.* 212:1522–23
37. Stuart-Harris, C. 1971. *Brit. Med. J.* 1: 387–88
38. Tilles, J. G. 1971. *N. Engl. J. Med.* 285:1260–61
39. O'Donoghue, J. M., Ray, C. G., Daniel, W. T. Jr., Beaty, H. N. 1973. *Am. J. Epidemiol.* 97:276–82
40. Kaplan, A. S., Lamar, B.-P., Kamiya, T. 1965. *Ann. NY Acad. Sci.* 130: 226–39
41. Calabresi, P. et al 1961. *Cancer Res.* 21:550–59
42. Renis, H. E., Buthala, D. A. 1965. *Ann. NY Acad. Sci.* 130:343–54
43. Kaufman, H. E. 1962. *Proc. Soc. Exp. Biol. Med.* 109:251–52
44. Swyers, J. S., Lausch, R. N., Kaufman, H. E. 1967. *Brit. J. Ophthalmol.* 51: 843–46
45. Jawetz, R., Coleman, V. R., Dawson, C. R., Thygeson, P. 1970. *Ann. NY Acad. Sci.* 173:282–91
46. Gurwith, M. J., Harman, C. E., Merigan, T. C. 1971. *Calif. Med.* 115:63–71
47. Gilbert, D. N., Johnson, M. T., Luby, J. P., Sanford, J. P. *Medicine.* In press
48. Nolan, D. C., Carruthers, M. M., Lerner, A. M. 1970. *N. Engl. J. Med.* 282:10–13
49. Nolan, D. C., Lauter, C. B., Lerner, A. M. 1973. *Ann. Intern. Med.* 78:243–46
50. Kibrick, S., Katz, A. S. 1970. *Ann. NY Acad. Sci.* 173:83–89
51. Juel-Jensen, B. E., MacCallum, F. O., Mackenzie, A. M. R., Pike, M. C. 1970. *Brit. Med. J.* 4:776–80
52. Barton, B. W., Tobin, J. O'H. 1970. *Ann. NY Acad. Sci.* 173:90–95
53. Lerner, A. M., Bailey, E. J. 1972. *J. Clin. Invest.* 51:45–49
54. Buthala, D. A. 1964. *Proc. Soc. Exp. Biol. Med.* 115:69–77
55. Rapp, F. 1964. *J. Immunol.* 93:6438
56. McCracken, G. H., Luby, J. P. 1972. *Pediatrics* 80:488–94
57. Ho, D. H. W., Frei, E. 1971. *Clin. Pharmacol. Ther.* 12:944–54
58. Hall, T. C. et al 1969. *Trans. Assoc. Am. Physicians* 82:210–12
59. McKenvey, E. M., Kwaan, H. C. 1969. *Blood* 34:706–11
60. Prager, D., Bruder, M., Sawitsky, A. 1971. *J. Pediat.* 78:321–23
61. Wilfert, C. M. 1971. *J. Pediat.* 79: 172–73
62. Juel-Jensen, B. E., MacCallum, F. O. 1972. *Herpes Simplex, Varicella and Zoster. Clinical Manifestations and Treatment.* Philadelphia: Lippincott
63. Pierce, L. E., Jenkins, R. B. 1973. *Arch. Ophthalmol.* 89:21–24
64. Stevens, D. A., Merigan, T. C. 1972. *J. Pediat.* 81:562–65
65. Davis, C. M., VanDersarl, J. V., Coltman, C. A. Jr. 1973. *J. Am. Med. Assoc.* 224:122–23
66. Hryniuk, W., Foerster, J., Shojania, M., Chow, A. 1972. *J. Am. Med. Assoc.* 219:715–18
67. Mankad, V. N., Gershon, A. A., Brunell, P. A. 1973. *J. Infec. Dis.* 127: 442–44
68. Kraybill, E. N. et al 1972. *J. Pediat.* 80:485–87
69. Plotkin, S. A., Stetler, H. 1970. *Antimicrob. Ag. Chemother.* 9:372–79
70. Talley, R. W., O'Bryan, R. M., Tucker, W. G., Loo, R. V. 1967. *Cancer* 20: 809–16
71. Holtermann, O. A., Nordin, A. A. 1969. *Proc. Soc. Exp. Biol. Med.* 132: 1003
72. Benedict, W. F., Karon, M. 1971. *Science* 171:680–82
73. O'Nell, F. J., Rapp, F. 1971. *J. Virol.* 7:692–95
74. Privat de Garilhe, M., De Rudder, J. 1964. *C. R. Acad. Sci.* 259:2725–28
75. Schabel, F. M. Jr. 1968. *Chemotherapy* 13:321–28
76. Sidwell, R. W., Arnett, G., Dixon, G. J. 1967. Abstracts of Papers, 7th Interscience Conf., Antimicrob. Ag. Chemother., Chicago, 1967
77. Miller, F. A. et al 1969. *Antimicrob. Ag. Chemother.* 8:136–47
78. Person, D. A., Sheridan, P. J., Herrmann, E. C. Jr. 1970. *Infec. Immunity* 2:815–20
79. Johnson, M. T., Luby, J. P., Buchanan, R. A. Unpublished
80. Furth, J., Cohen, S. S. 1968. *Cancer Res.* 28:2061–67
81. Brink, J. J., LePage, F. A. 1964. *Cancer Res.* 24:312–18
82. York, J. L., LePage, G. A. 1966. *Can. J. Biochem.* 44:19–26
83. Furth, J. J., Cohen, S. S. 1967. *Cancer Res.* 27:1528–33
84. Moore, E. C., Cohen, S. S. 1967. *J. Biol. Chem.* 242:2116–18
85. Wagar, M. A., Burgoyne, L. A., Atkinson, M. R. 1971. *Biochem. J.* 121:803–9
86. Miller, F. A., Sloan, B. J., Silverman, C. A. 1970. *Antimicrob. Ag. Chemother.* 9:192–95

87. Glazko, A. J., Mourer, D. R., Chang, T. Unpublished
88. Pavan-Langston, D., Dohlman, C. H. 1972. *Am. J. Ophthalmol.* 74:81–88
89. Ch'ien, L. T., Cannon, N. J., Dismukes, W. E., Buchanan, R. A., Alford, C. A. 1973. *Clin. Res.* 21:61
90. Nichols, W. W. 1964. *Cancer Res.* 24: 1502–6
91. Wilkerson, S., Finley, S. C., Finley, W. H., Ch'ien, L. T. 1973. *Clin. Res.* 21:52
92. Bauer, D. J. 1955. *Brit. J. Exp. Pathol.* 36:105–14
93. Bauer, D. J., Sadler, P. W. 1960. *Brit. J. Pharmacol.* 15:101–10
94. Bauer, D. J., St. Vincent, L., Kempe, C. H., Downie, A. W. 1963. *Lancet* 2: 494–96
95. Rao, A. R. et al 1969. *Indian J. Med. Res.* 57:477–83
96. Do Valle, L. A. R. et al 1965. *Lancet* 2:976–78
97. Heiner, G. G. et al 1971. *Am. J. Epidemiol.* 94:435–49
98. Adels, B. R., Oppe, T. E. 1966. *Lancet* 1:18–20
99. Isaacs, A., Lindenmann, J. 1957. *Proc. Roy. Soc. (Biol.)* 147:258–67
100. Glasgow, L. A. 1965. *J. Pediat.* 67: 104–12
101. Wagner, R. R. 1965. *Am. J. Med.* 38: 726–37
102. Wheelock, E. F., Larke, R. P. B., Caroline, N. L. 1968. *Progr. Med. Virol.* 10: 286–347
103. Vilcek, J. 1969. *Interferon.* West Berlin:Springer-Verlag. 141 pp.
104. Merigan, T. C. 1970. *Arch. Intern. Med.* 126:49–157
105. Stevens, D. A., Merigan, T. C. 1970. *J. Clin. Invest.* 51:1170–78
106. Soloviev, V. D. 1969. *Bull. WHO* 41: 683–88
107. Stringfellow, D. A., Glasgow, L. A. 1972. *Infec. Immunity* 6:743–47
108. Hilleman, M. R. 1970. *J. Infec. Dis.* 121:196–211
109. Guerra, R., Frezzotti, R., Bonanni, R., Dianzani, F., Rita, G. 1970. *Ann. NY Acad. Sci.* 173:823–30
110. Park, J., Baron, S. 1968. *Science* 162: 811–13

Copyright 1974. All rights reserved

CLINICAL UTILITY OF IN VITRO EVALUATION OF GRANULOPOIESIS[1]

❖7111

Peter L. Greenberg, M.D. and Stanley L. Schrier, M.D.
Departments of Medicine, Veterans Administration Hospital, Palo Alto, California, and Stanford University School of Medicine, Stanford, California

INTRODUCTION

The narrow range of neutrophils normally present in the peripheral blood of man and experimental animals indicates a rigorous control of granulopoiesis, perhaps via feedback regulatory mechanisms. Abnormalities of these control mechanisms would logically account for variations from normal in patients with disorders of granulopoiesis. To date, clinical studies, animal experimentation, and isotopic leukokinetic investigations have been utilized to obtain information regarding granulopoiesis. These experimental approaches have shown that neutropenia results in the appearance of humoral factors in blood which enhance granulocyte production (1, 2) and cause the release of mature granulocytes from the marrow granulocyte reserve compartment (3–5). Leukokinetic studies have defined granulocytic compartmental flux rates, compartment sizes, and cell transit times (6, 7). The concept has evolved that mature blood granulocytes are generated by the proliferative activity of less mature, but morphologically identifiable marrow precursors (myeloblasts, promyelocytes, and myelocytes), and that these cells are derived from cells committed to granulocytic differentiation. These granulocytic progenitor cells (GPC) are the clonal progeny of pluripotential hemopoietic stem cells (8, 9) regulated by inductive microenvironmental influences present within hemopoietic tissues (10, 11).

No direct way of measuring the GPC compartment or its humoral stimuli was available, however, until an in vitro marrow culture technique was developed which permitted quantitation of murine GPC proliferation and differentiation (12, 13).

---

[1] This study was supported by grants from the National Institutes of Health, National Cancer Institute CA13141, the American Cancer Society (California Division), and Veterans Administration research funds. Dr. Greenberg is a Scholar of the Leukemia Society of America.

Under the necessary influence of a specific humoral regulatory substance called colony stimulating factor (CSF), the progeny of these GPC form colonies of granulocytic and macrophagic cells in soft agar or methyl cellulose gels. CSF is provided by cell feeder layers (12, 13), conditioned medium from cell cultures (14–16), and various tissues (17–20), and is present in serum (21, 22) and excreted into the urine (23–25). Provision of CSF by human cell sources, most commonly from blood leukocytes or spleen, has permitted in vitro growth of human GPC (26, 27). Monocytes have been shown to be the predominant blood leukocyte source of CSF (28, 28a). In this review we focus on the contributions to the understanding of clinical disease made by studies in which the granulocytic colony forming capacity (CFC) of human marrow and the CSF in human serum and urine were measured and correlated with disorders of granulopoiesis.

MEASUREMENT OF COLONY FORMING CAPACITY

CFC data in man are generally reported as cloning efficiency (i.e. colonies/10^5 marrow cells plated), whereas in mice more precise quantitation has been achieved by determining the colony forming cells present within a defined portion of marrow (i.e. colonies/femur). This measurement of GPC concentration in human marrow is affected by variations in marrow distribution patterns, marrow cell differential counts, and dilution of marrow sample by peripheral blood. These variations limit the use of measurement of GPC concentration for quantitating granulopoiesis.

Some recent refinements have improved our ability to characterize GPC responsiveness to demand for new granulocytes, and have led to better functional and morphologic identification of GPC. The proliferative status of GPC has been measured by the thymidine suicide technique, which determines the proportion of GPC in the DNA synthesis phase of the cell cycle (29). This method provides a more representative measure of human marrow granulopoietic responsiveness, since results of these studies are not altered by the above-mentioned factors. Cell separation techniques, using velocity sedimentation and density gradient procedures, have allowed for considerable concentration of GPC. Velocity sedimentation has shown that human colony forming cells are equivalent in size to mouse GPC (30). Relatively homogeneous populations of monkey granulocytic progenitor cells have been obtained by density gradient cell separation procedures, and the in vitro colony forming cell has been shown to be a mononuclear cell, 9–11 μ in diameter, with agranular basophilic cytoplasm and a leptochromatic nucleus, suggesting a transitional lymphocyte (31). Single-cell cloning experiments have indicated that the GPC gives rise to both granulocytes and macrophages in culture (31).

MEASUREMENT OF COLONY STIMULATING FACTOR

CSF is needed continuously for sustaining cellular proliferation and differentiation in vitro (32), and causes an increase in proportion of GPC in DNA synthesis (33). CSF has been characterized biochemically as a glycoprotein with mol wt of approximately 45,000 (23). Tissue and humoral sources of CSF, although having similar biologic and antigenic properties, vary in their molecular size and charge (17–20, 34). Human urinary CSF is stimulatory for mouse but not human marrow. The numerous sources of CSF suggest that either a number of different cells provide CSF or that a widely distributed cell or tissue, common to many organs, is responsible for CSF production.

Nondialyzable lipoprotein substances are present in serum and urine and inhibit the action of CSF in vitro (35). These inhibitors, in addition to decreasing colony formation, cause premature macrophage transformation of the in vitro colonies (36). Following radiation, serum inhibitor levels fall concomitant with marrow regeneration (37). Preliminary evidence has suggested that CSF inhibitors may depress the capacity of cells to produce CSF (38). However, these inhibitory effects have been demonstrated in mouse but not human cells. The cellular source and possible role of this inhibitor as a modifier of granulopoietic stimuli remain to be determined. Low molecular weight dialyzable inhibitory substances, chalones (39), have been described. However, the inability to detect these materials in serum and their lack of specificity for inhibiting granulopoiesis have cast doubt on their biological significance (40). A recently developed method for concentrating CSF permits measurement of CSF levels essentially free of both types of inhibitory substances (41), and has provided a means for improved quantitation of CSF.

PHYSIOLOGICAL RELEVANCE OF IN VITRO PARAMETERS OF GRANULOPOIESIS

Following irradiation, cytotoxic drug exposure, and endotoxin administration to mice, the granulocytic colony forming capacity (CFC) is altered (1, 42, 43), and levels of serum CSF (44–46) and provision of CSF by marrow cellular sources (37) are increased. Expansion of the granulocytic progenitor cell and myeloblast-promyelocyte compartments occurs following irradiation (42), concomitant with neutropenia and serum CSF elevation (44). In response to demand for new granulocytic cells, caused by irradiation or endotoxin administration, a marked increase in the proportion of stem cells and GPC in DNA synthesis occurs (29, 45, 46). Endotoxin-induced CSF elevations precede a wave of granulopoiesis, in which increased proportions of stem cells, GPC, and recognizable early myeloid precursors enter DNA synthesis (43, 46). Injection of partially purified human urine CSF preparations into mice causes an increase in the size of the CFC and myeloblast-promyelocyte compartments and a subsequent rise of labeled blood granulocytes (47). Present evidence indicates that CSF elaboration arises through tissue breakdown and/or antigenic stimulation by foreign or endogenous proteins, such as endotoxin, and not because of neutropenia per se (19, 43, 45, 48). Elevated levels of serum and urine CSF have been found in patients with bacterial and viral infections, various neoplasms, and myeloproliferative disorders (22, 49). The granulocytic proliferative changes occurring subsequent to injection of CSF or induced elevation of CSF have led to the proposal that this substance may be a physiologic regulator of granulopoiesis and monocyte formation. However, a word of caution is necessary. Inflammation, tissue breakdown, and exposure to endotoxin, radiation, or impure CSF preparations produce numerous and complex events, including the elaboration of materials that tend to mask the specificity of the action of CSF. Sustained granulopoietic effects resulting from more highly purified CSF preparations are needed before the physiologic role of CSF can be established.

APPLICATION TO HUMAN DISORDERS OF GRANULOPOIESIS

Neutropenic Disorders

These in vitro techniques have been applied to the study of patients with neutropenia. The granulopoietic response to neutropenic demand appears to be dependent on the etiology of the disorder (50). An inverse relationship exists between the percentage of marrow GPC in DNA synthesis and the blood neutrophil count, thus supporting the hypothesis (51) that negative feedback regulation of granulopoiesis exists in vivo. In those neutropenic patients with splenomegaly, where it could be anticipated that increased granulocytic turnover as well as normally responsive GPC existed, the expected increases occurred in both marrow CFC and the percentage of GPC in DNA synthesis (50). Thus, the neutropenia present in these patients appeared to be due mainly to accelerated neutrophil removal. The finding of an elevated percentage and concentration of marrow GPC in DNA synthesis in patients with megaloblastic anemia is consistent with the suggestion that the neutropenia in these patients may be due to ineffective granulopoiesis (52).

Decreased production appears to contribute significantly to the neutropenia present in patients with myeloid hypoplasia, Felty's syndrome, and idiopathic neutropenia (50). In myeloid hypoplasia there is deficient concentration of GPC present in the marrow specimens, since markedly decreased CFC values were observed even when these values were corrected for the number of granulocytic precursors plated. Therefore, in myeloid hypoplasia there is a low number of morphologically identifiable myeloid cells in the marrow and a decreased ability of those myeloid cells present to proliferate and form colonies in vitro. Decreased CFC in marrow of other patients with aplastic anemia has also been reported (53).

The CFC and the proportion of GPC in DNA synthesis in patients with Felty's syndrome were found to be either decreased or not different from those of control subjects (50). These findings, showing in vitro production defects in these patients, are consistent with leukokinetic measurements (54) indicating that most patients with Felty's syndrome had normal or decreased granulocytic turnover rates associated with rapid peripheral removal of neutrophils. These data, along with the variable results obtained with splenectomy, indicate that the reduced neutrophil levels in this disorder result from a combination of deficient production and increased peripheral removal of granulocytes.

The in vitro marrow proliferative characteristics of patients with idiopathic neutropenia are complex (50). This group of patients has been classified as having chronic stable neutropenia, normal spleen size, and no evidence for systemic disorders or toxic drug ingestion, with normal marrow granulocytic cellularity and morphology. A relatively wide spread of in vitro proliferative values was found in these subjects, suggesting that this category was comprised of a heterogeneous group of patients. In idiopathic neutropenia, as a group, there was no significant difference of CFC or the proportion of GPC in DNA synthesis from control subjects who had normal neutrophil counts, thereby suggesting that these patients exhibited suboptimal granulopoietic compensation. The in vitro proliferative heterogeneity present in neutropenic patients classified as idiopathic may reflect our inability to define clinically the factors necessary for categorizing this patient group. This interpretation is supported by leukokinetic data (54) indicating that patients with idiopathic neutropenia have a wide range of granulocytic turnover rates and blood neutrophil survival times.

Studies in Yemenite Jews with the genetic ethnic form of neutropenia, whose illness would otherwise fit into the category of idiopathic or chronic benign neutropenia, had increased CFC, suggesting that deficient production was not the cause for their abnormality (55). This result was in contrast to findings obtained with infantile genetic agranulocytosis (Kostmann's syndrome) where the marrow cloning efficiency was similar to normal controls (56). Despite the lack of marrow granulocytic maturation beyond the promyelocyte or myelocyte level in this patient, normally maturing granulocytic colonies developed in vitro when provided with CSF. Thus the morphologic pattern of marrow maturation arrest seen in these patients, and in other patients with similar in vitro findings (50, 57, 58), indicates that since the granulocytes in these disorders were not intrinsically incapable of maturation, there may be a deficiency of a humoral inducer of granulopoiesis. These findings do not exclude the possibility that factors inhibiting granulopoiesis in vivo were inactive in vitro. However, in another patient with congenital agranulocytosis, the abnormal in vivo granulocytic maturation pattern was mimicked by the morphology of the in vitro colonies (59). It is possible that these discrepant findings may be due to differing clinical categorization of these patients.

Cyclic neutropenia is a disorder characterized by regularly recurrent episodes of neutropenia which generally occur every 19–24 days. This periodicity suggests that an active feedback control system regulates granulopoiesis and has provided a model (51) for evaluating such regulatory factors. A study of a patient with cyclic neutropenia showed that marrow proliferative activity (CFC and the proportion of GPC in DNA synthesis) was inversely related to his peripheral neutrophil count (50). Serial measurements indicated that serum and urine CSF levels in this patient also varied inversely with the peripheral neutrophil count and directly with the monocyte count (60). Humoral substances inhibiting granulopoiesis were absent throughout the neutrophil cycle. Endotoxemia was shown to be a possible mediator for the elaboration of CSF in this patient. These investigations, plus the results of plasma infusion studies, suggest that cyclic neutropenia is due to a quantitative decrease of stem cells or GPC input into granulopoiesis, causing subsequent intermittent decreases in the size of the marrow granulocyte reserve (60).

In a previous report of a patient with cyclic neutropenia (61), there was a different interpretation of the data, but review of the charts indicates that two of three peak CSF levels occurred during periods of marked neutropenia. The reciprocal relationship between CSF and neutrophil levels also occurs in gray collie dogs with cyclic neutropenia (62). Defective granulocyte production has been shown to cause drug-induced (63) and genetic (64) canine cyclic neutropenia.

These studies support the model of oscillatory granulopoiesis (51, 63), which indicates that in subjects lacking granulocyte reserves the rate of granulopoiesis is solely dependent on the slope of a computed curve relating marrow granulocyte production rate to the peripheral neutrophil level. At very neutropenic levels, when the marrow granulocyte reserve is lacking, the slope of this curve is quite steep and granulopoiesis is increased. This intermittent relative enhancement of production may result from the undampened intensity of the granulopoietic stimulus occurring in the absence of marrow granulocyte reserves. The switching on and off of production would lead to the pattern of cyclic neutropenia.

The in vitro parameters of granulopoiesis are perturbed following chemotherapy in mice (1, 46, 65) and in man (66, 66a). The sensitivity of mouse hemopoietic stem cells and GPC to cytotoxic agents is dependent upon their proliferative state, with marrows containing a high proportion of these cells in DNA synthesis more susceptible to the toxic effects of cycle-specific drugs (46). In patients undergoing chemotherapy with such agents, marked alterations of CFC, the proportion of GPC in DNA synthesis, and CSF output occur (66, 66a). An initial decline of GPC in DNA synthesis was followed by a sharp increase of these cells 1–2 days following therapy, associated with increased urinary output of CSF. Treatment given when a high proportion of the GPC was in DNA synthesis was followed by neutropenic toxicity (66). These studies have proven useful for indicating the oscillatory pattern of marrow granulopoietic compensation in response to cytotoxic treatment in mouse and man, providing a basis for determining the efficacy with which cytotoxic drugs destroy proliferative activity of GPC, and providing a means for more directly assessing the potential for hemopoietic toxicity.

Leukemia and Other Myeloproliferative Disorders

In vitro indices of granulopoiesis are markedly altered in leukemia and other myeloproliferative disorders (26, 67–74). In acute myeloid leukemia (AML), CFC is abnormal, with either no colony formation or the formation of excessive numbers of small abortive colonies or clusters (26, 70, 71, 73). The leukemic colony forming cells in AML are of abnormally light buoyant density, and a low proportion of these cells are in DNA synthesis. Remission in AML is associated with a return to normal in colony size and number, and of GPC density and cell cycle status (70, 73). The progeny of leukemic myeloblasts in the peripheral blood generally have markedly deficient ability to provide CSF. However, during remission the leukocytes can provide normal amounts of CSF (70, 75). Karyotypic analyses have indicated that the granulocytic colonies and clusters which developed in culture from marrow of AML patients in relapse with aneuploidy were derived from representative cells of the leukemic population (76). In some patients with cytogenetic abnormalities, remission was associated with restoration of normal cytogenetic status both in direct marrow preparations and from the cells in developing agar colonies (76).

This technique thus appears to be able to detect and distinctively characterize normal and leukemic cells. These findings suggest that AML marrow consists of coexisting populations of normal and leukemic cells, with normal clones emerging in remission and a leukemic clone predominating in relapse. Persisting normal GPC thus appear to lie dormant amid the expanding leukemic population. The mechanism whereby normal clones are rendered dormant is not known. To date, experiments attempting to show inhibition of normal CFC by leukemic cells or serum have not been successful (67, 70).

The cells within the colonies from leukemic marrows are capable of in vitro differentiation to mature appearing neutrophils (26, 69, 70, 73). The leukemic colony forming cells that differentiate and form colonies are not autonomous in vitro, since they require the provision of CSF in order to proliferate (70, 73, 75). These findings, if applicable to the in vivo situation in AML, cause revision of some previously held concepts about the autonomous and nonmaturing nature of leukemic cells. Sequential studies have indicated that marrow CFC and colony forming cell density distribution patterns correlate with and may precede detectable changes in clinical and morphologic status by weeks or months. Therefore these in vitro measurements appear to be useful for delineating the completeness of clinical remissions by quantifying the remaining functionally normal granulocytic cells (70, 73).

Patients with chronic myeloid leukemia (CML) have greatly increased CFC, particularly in the peripheral blood, with the colony forming cells having abnormally light buoyant density (73). As these patients evolve into the acute blastic transformation phase of their disease, the same abnormal growth pattern occurs as that seen in AML in relapse (73). In cultures from patients with Philadelphia chromosome (Ph_1) positive CML, the colonies contain Ph_1 chromosomes in all (76) or the majority (77) of the cells, suggesting the growth of leukemic cells. The clonal nature of these colonies was indicated in the latter study by the lack of mixing of Ph_1 positive and negative cells within the colonies.

These in vitro studies have been employed for evaluating patients with preleukemia (70, 78). This disorder comprises a group of patients with refractory anemia, neutropenia, thrombopenia, or monocytosis associated with defective myeloid maturation, and with an enhanced tendency to evolve into AML (79, 80). The disordered marrow CFC and inability of peripheral leukocytes to provide CSF in vitro are as abnormal as the pattern seen in patients with AML in relapse (70). Preliminary data suggest that the cluster forming cells in some of these patients are of abnormally light density and have abnormal cell cycle proliferative status (38). When viewed in the context of the chronicity of their clinical courses, and in the absence of morphologic evidence for leukemic change, these abnormalities suggest either that preleukemic marrow is populated by a potentially leukemic clone that has greater capacity for differentiation in vivo than a frankly leukemic clone, or that normal and leukemic clones coexist and the preleukemic host is somehow able to cope with the abnormal line for an extended time.

Urinary CSF output varies depending on the type of leukemia and clinical status of the patient. In the majority of patients with AML, at diagnosis or in relapse, low or absent urinary CSF levels were found (24). As these patients entered drug-induced remission, CSF levels rose to normal or very high levels, only to fall again to subnormal levels with relapse. In other studies where disease status or treatment was not considered, variable serum and urine CSF levels were found (49). The low CSF levels found in these early studies need to be re-evaluated, since the recent technical modification (41), which eliminates lipoprotein inhibitors of CSF, was not utilized. Patients with CML and preleukemia have high levels of urinary CSF excretion, and these levels decrease as the patients evolve into AML (22, 38). CSF production by marrow decreases after neoplastic infiltration of mouse marrow (38). These findings suggest that the ability of the marrow environment to support or stimulate granulopoiesis becomes defective with evolution of these diseases. Since increases of CSF may be present in patients and animals with preleukemia, the possibility has been raised as to whether such an enhanced proliferative stimulus increases the susceptibility of potentially leukemic cells to become overtly leukemic (38). An alternative explanation of these findings is that the elevated CSF levels in preleukemia and CML are due to the presence of antigenic stimulation of hosts (? by neoantigens, oncogenic viruses, etc) that retain responsive CSF-producing cells. Progression of disease may diminish this CSF-producing cellular population or its responsiveness, and thus decrease the marrow environment's ability to support normal granulopoiesis.

SUMMARY

The in vitro techniques described have contributed to the overall understanding of granulopoiesis by providing quantitative methods for evaluating granulopoietic responsiveness of marrow and its humoral proliferative stimuli. The physiological relevance of CSF and its inhibitors needs to be further clarified. In the evolution of pathologic states, the methods used indicate that the degree of marrow compensation for sustained peripheral neutropenia varies depending on the etiology of the neutropenia. Experimental data in AML have provided evidence for the biclonal hypothesis of marrow in that disorder by detecting and distinctively characterizing normal and leukemic granulocytic precursors, and have more clearly defined preleukemic disorders. The completeness of remission in AML appears to be evaluated more effectively by marrow culture techniques than by standard morphologic methods.

PERSPECTIVES

Further purification of CSF and development of a radioimmunoassay will provide useful information regarding the tissues of origin, mechanism of action, and stimulus for production of CSF. Cell separation techniques, which concentrate both GPC and CSF-producing cells, should result in real advances, since relatively homogeneous populations of GPC will lend themselves to better quantification and provide more defined populations with which to study cell-to-cell interaction, susceptibility to drug action, oncogenic agents, and CSF.

Parallel studies of marrow granulopoiesis using the clonal techniques described, liquid marrow culture with the Marbrook chamber (81), and implanting marrow cells in vivo in diffusion chambers (82) should provide the kinds of correlative information not available with any single technique for evaluation of granulocyte production. This combination of techniques should further contribute to our understanding of pathogenetic mechanisms in human disorders of granulopoiesis.

Literature Cited

1. Breivik, H. 1972. *J. Cell Physiol.* 79:171
2. Rothstein, G., Hugl, E., Bishop, C., Athens, J., Ashenbrucker, H. 1971. *J. Clin. Invest.* 50:2004
3. Katz, R., Gordon, A., Lapin, D. 1966. *J. Reticuloendothel. Soc.* 3:103
4. Boggs, D., Marsh, J., Chervenick, P., Cartwright, G., Wintrobe, M. 1968. *Proc. Soc. Exp. Biol. Med.* 127:689
5. Marsh, J., Levitt, M. 1971. *Blood* 27: 647
6. Cartwright, G., Athens, J., Wintrobe, M. 1964. *Blood* 24:780
7. Cronkite, E., Vincent, P. 1970. *Hemopoietic Cellular Proliferation,* ed. F. Stohlman Jr., 211–28. New York: Grune & Stratton
8. Wu, A. M. et al 1968. *Proc. Nat. Acad. Sci. USA* 59:1209–15
9. Rickard, K. A. et al. See Ref. 7, p. 238–48
10. McCulloch, E., Till, J. See Ref. 7, p. 15–25
11. Trentin, J. 1971. *Am. J. Pathol.* 65: 621
12. Bradley, T. K., Metcalf, D. 1966. *Aust. J. Exp. Biol. Med. Sci.* 44:287
13. Pluznik, D. H., Sachs, L. 1965. *J. Cell. Comp. Physiol.* 66:319
14. Pluznik, D. H., Sachs, L. 1966. *Exp. Cell Res.* 43:553
15. Bradley, T. R., Sumner, M. A. 1968. *Aust. J. Exp. Biol. Med. Sci.* 46:607
16. Chervenick, P. A., Boggs, D. R. 1970. *Science* 169:691
17. Bradley, T., Stanley, E. R., Sumner, M. 1971. *Aust. J. Exp. Biol. Med. Sci.* 49:595
18. Stanley, E. R., Sheridan, J. 1971. *J. Cell. Physiol.* 78:451
19. Sheridan, J., Metcalf, D. 1972. *J. Cell. Physiol.* 80:129
20. Austin, P., McCulloch, E., Till, J. 1972. *J. Cell. Physiol.* 79:181
21. Robinson, W. A., Metcalf, D., Bradley, T. R. 1967. *J. Cell. Physiol.* 69:83
22. Foster, R., Metcalf, D., Robinson, W. A., Bradley, T. R. 1968. *Brit. J. Haematol.* 15:147
23. Stanley, E. R., Metcalf, D. 1969. *Aust. J. Exp. Biol. Med. Sci.* 47:467
24. Robinson, W. A., Pike, B. L. 1970. *N. Engl. J. Med.* 282:1291
25. Chan, S. 1970. *Proc. Soc. Exp. Biol. Med.* 134:733
26. Pike, B. L., Robinson, W. A. 1970. *J. Cell. Physiol.* 76:77
27. Paran, M., Sachs, L., Barak, Y., Resnitzky, P. 1970. *Proc. Nat. Acad. Sci. USA* 67:1542
28. Golde, D., Cline, M. 1972. *J. Clin. Invest.* 51:2981
28a. Chervenick, P., Lobuglio, A. 1972. *Science* 178:164
29. Iscove, N., Till, J., McCulloch, E. 1970. *Proc. Soc. Exp. Biol. Med.* 134:33
30. Iscove, N. N., Messner, H., Till, J. E., McCulloch, E. A. 1972. *Ser. Haematol.* 2:37
31. Moore, M. A. S., Williams, N., Metcalf, D. 1972. *J. Cell. Physiol.* 79:283
32. Metcalf, D. 1970. *J. Cell. Physiol.* 76:89
33. Moore, M.A.S. *Proc. 2nd Int. Workshop Hemopoiesis Culture,* Warrenton, Virginia. In press
34. Stanley, E. R., Metcalf, D. 1971. *Proc. Soc. Exp. Biol. Med.* 137:1029
35. Chan, S., Metcalf, D., Stanley, E. R. 1971. *Brit. J. Haematol.* 20:329
36. Chan, S. H. 1971. *Aust. J. Exp. Biol. Med. Sci.* 49:553
37. Chan, S. H., Metcalf, D. 1973. *Cell Tissue Kinet.* 6:188
38. Metcalf, D. 1973. *Brit. J. Cancer* 27:191
39. Benestad, H., Rytomaa, T., Kiviniemi, K. 1973. *Cell Tissue Kinet.* 6:147
40. Metcalf, D. 1971. *Aust. J. Exp. Biol. Med. Sci.* 49:351
41. Stanley, E. R., Metcalf, D., Maritz, J., Yeo, G. 1972. *J. Lab. Clin. Med.* 79:657
42. Rickard, K. A., Morley, A., Howard, D., Stohlman, F. Jr. 1971. *Blood* 37:6
43. Quesenberry, P. et al 1973. *Blood* 41:391
44. Morley, A., Rickard, K., Howard, D., Stohlman, F. Jr. 1971. *Blood* 37:14
45. Quesenberry, P. J. et al 1972. *N. Engl. J. Med.* 286:227
46. Eaves, A., Bruce, W. 1972. *Serv. Haematol.* 2:64
47. Metcalf, D., Stanley, E. R. 1971. *Brit. J. Haematol.* 21:481
48. Morley, A., Quesenberry, P., Bealmear, P., Stohlman, F., Wilson, R. 1972. *Proc. Soc. Exp. Biol. Med.* 140:478
49. Metcalf, D., Chan, S., Gunz, F., Vincent, P., Ravich, R. 1971. *Blood* 38:143
50. Greenberg, P. L., Schrier, S. L. 1973. *Blood* 41:753
51. King-Smith, E., Morley, A. 1970. *Blood* 36:254
52. Perillie, P., Kaplan, S., Finch, S. 1967. *N. Engl. J. Med.* 277:10
53. Kurnick, J. E., Robinson, W. A., Dickey, C. A. 1971. *Proc. Soc. Exp. Biol. Med.* 137:917
54. Bishop, C., Rothstein, G., Ashenbrucker, H., Athens, J. W. 1971. *J. Clin. Invest.* 50:1678

55. Mintz, U., Sacks, L. 1973. *Blood* 41:745
56. Barak, Y., Paran, M., Levin, S., Sachs, L. 1971. *Blood* 38:74
57. Zuelzer, W., Bajoghli, M. 1964. *Blood* 23:359
58. Lau, P., Brody, J., Beizer, L. 1967. *Blood* 29:462
59. L'Esperance, P., Brunning, R., Good, R. 1973. *Proc. Nat. Acad. Sci. USA* 70:669
60. Greenberg, P., Bax, I., Schrier, S., Levin, J. Submitted for publication
61. Mangalik, A., Robinson, W. A. 1973. *Blood* 41:79
62. Dale, D., Brown, C., Carbone, P., Wolff, S. 1971. *Science* 173:152
63. Morley, A., Stohlman, F. 1970. *N. Engl. J. Med.* 282:643
64. Dale, D., Alling, D., Wolff, S. 1972. *J. Clin. Invest.* 51:2197
65. Brown, C., Carbone, P. 1971. *Cancer Res.* 31:185
66. Greenberg, P., Bax, I., Mara, B., Schrier, S. Submitted for publication

66a. Greenberg, P. L., Schrier, S. L. 1972. *Proc. Am. Soc. Hematol. Meet. Dec. 1972*, p. 134

67. Senn, J., McCulloch, E., Till, J. 1967. *Lancet* 2:597
68. Harris, J. E., Freireich, E. J. 1970. *Blood* 36:61
69. Paran, M., Sachs, L., Barak, Y., Resnitsky, P. 1970. *Proc. Nat. Acad. Sci. USA* 67:1542
70. Greenberg, P. L., Nichols, W. C., Schrier, S. L. 1971. *N. Engl. J. Med.* 284:1225
71. Iscove, N. N., Senn, J. S., Till, J. E., McCulloch, E. A. 1971. *Blood* 37:1
72. Brown, C. H., Carbone, P. P. 1971. *J. Nat. Cancer Inst.* 46:989
73. Moore, M. A. S., Williams, N., Metcalf, D. 1973. *J. Nat. Cancer Inst.* 50:603
74. Moore, M. A. S. *Proc. 2nd Int. Workshop Hemopoiesis Culture,* Warrenton, Virginia. In press
75. Moore, M., Williams, N., Metcalf, D. 1973. *J. Nat. Cancer Inst.* 50:591
76. Moore, M. A. S., Metcalf, D. 1973. *Int. J. Cancer* 11:143
77. Chervenick, P., Ellis, L., Pan, S., Lawson, A. 1971. *Science* 174:1134
78. Senn, J., Pinkerton, P. 1972. *Brit. J. Haematol.* 23:277
79. Block, M., Jacobson, L. D., Bethard, M. F. 1953. *J. Am. Med. Assoc.* 152:1018
80. Linman, J. W. 1970. *J. Chronic Dis.* 22:713
81. Golde, D., Cline, M. J. 1973. *N. Engl. J. Med.* 288:1083
82. Boyum, A., Boecker, W., Carsten, A., Cronkite, W. 1972. *Blood* 40:163

Copyright 1974. All rights reserved

CRYSTAL DEPOSITION JOINT DISEASE

❖7112

Daniel J. McCarty, M.D.
Section of Arthritis and Metabolism, University of Chicago, Pritzker School of Medicine, Chicago, Illinois

Inflammation of joints may be associated with leukocytic phagocytosis of either monosodium urate (MSU) or calcium pyrophosphate dihydrate (CPPD) microcrystals, and a previous review in this series summarized the mechanisms involved in "crystal induced inflammation" (1). The broader generic term "crystal deposition disease" was coined to embrace both classic gout (MSU crystal deposition) and pseudogout or chondrocalcinosis (CPPD crystal deposition) (2). With the recognition of the crystals as a sine qua non, the definition of these conditions was changed from being nonspecific and clinical to specific and crystallographic.

Such crystal deposits probably represent final common pathways in either urate or pyrophosphate metabolism. In the case of gout and MSU crystals, a number of primary and secondary biochemical abnormalities leading to hyperuricemia are known and still others are being sought. It is likely that CPPD deposition is also a result of one or more biochemical perturbations. Systemic hyperuricemia resulting from increased synthesis of urate in the liver or decreased excretion by the kidney, or an elevated joint fluid inorganic pyrophosphate (PP_i) level are viewed as necessary but not sufficient causes for formation of MSU or CPPD crystals respectively. This review will focus chiefly on PP_i metabolism and pseudogout as several good reviews of uric acid metabolism have appeared recently (3, 4). No attempt will be made at an exhaustive review of the clinical features of pseudogout. The reader is again referred to recent reviews on this subject (5, 6).

PREVALENCE OF CRYSTAL DEPOSITION DISEASES

The pathologic prevalence of articular CPPD crystals at the time of death has been estimated on the basis of a cadaver study at 3.2% (7). MSU crystal deposits were found in 2% of cadavers in the same study, which used the knees as reference joints.

The ratio of the clinical incidence of symptomatic gout to pseudogout is about 2/1.[1] It is likely that many, or even most, individuals with crystal deposits remain asymptomatic. This idea is readily confirmed in pseudogout, where most of the joints showing radiologic evidence of CPPD deposits are symptomless.

PATHOGENESIS OF CRYSTAL DEPOSITS IN JOINTS

Why MSU and CPPD crystals deposit selectively in avascular or nearly avascular cartilage and dense fibrous tissue is unknown. Katz & Schubert have shown that solutions of protein polysaccharide increase the solubility of monosodium urate, suggesting that increased degradative enzymatic activity in cartilage might lead to local supersaturation and mineral phase formation (8). Genetic factors have been clearly implicated in a series of cases studied in Czechoslovakia, Chile, and the Netherlands (9–11). Such cases show the same clinical features on physical and roentgenologic examination as do sporadic cases, but the disease tends to be more severe, with onset at an earlier age and more rapid progression. More important perhaps is the observation that familial cases show no association with the potpourri of other metabolic conditions found to be associated with CPPD, as discussed below. As no generation was skipped in any of these families, and as about half of the older offspring of an afflicted parent developed joint calcifications, a dominant hereditary pattern was suggested. No male to male transmission was recorded until an abstract outlining the recent Dutch experience was published, suggesting that the latter cases differ genetically from the others. In the Czechoslovakian and Chilean series a closed population and inbreeding were known to exist, so that "pseudodominance," a recessive trait appearing as a dominant, might be postulated.

Some clues to pathogenesis have been gleaned from a consideration of the associated diseases. Diabetes mellitus, hypertension, atherosclerosis, and mild azotemia may be more common in pseudogout than in the general population of the same age —the average age in our series of nearly 300 cases of pseudogout patients is 71 years (5). Hyperuricemia, found in 34%, and classic urate gout, found in 5% of pseudogout cases, appears excessive. Hyperparathyroidism, found in 7.2% of our series, and nephrolithiasis, found in 7.5%, also appear to represent a greater degree of association than might be expected by chance alone. The association with hemochromatosis has been described repeatedly (12–14). Two cases of associated mild asymptomatic hemochromatosis were recently described by Gordon & Little (15). We found that of 28 pseudogout cases, 2 had elevated serum iron levels and increased transferrin saturation (16), so it is likely that an association with hemochromatosis, often inapparent clinically, is fairly common. Other associations in our series include gynecomastia (7% of male cases), inflammatory small bowel disease (2.1%), Paget's disease of bone (3.4%), and psoriatic arthritis (4%). A rare but potentially important association with hypophosphatasia was reported recently by O'Duffy (17). Several cases of pseudogout have been reported in association with Wilson's disease, but proof of crystal identity has not been obtained (18, 19).

[1] McCarty, D. J., Unpublished data.

Recently, deposits of CPPD microcrystals in the thickened rheumatoid synovium of the knee joint of 3/43 consecutive patients undergoing total knee joint replacement were described by Bywaters (20). The articular cartilage in all had undergone extensive destruction precluding examination for crystals. In a controlled radiological study, Good & Rapp found calcifications of menisci in 8% of 38 cases of rheumatoid arthritis and in 5% of 43 patients with gout (21).

But by far the most striking association, as pointed out in our original report, is that with osteoarthritis (22). About 70% of pseudogout cases show a progressive chronic arthritis of one or both hips, knees, elbows, shoulders, wrists, and of the metacarpophalangeal (MCP) joints. Except for superimposed acute or subacute inflammatory attacks and its peculiar distribution, the chronic arthritis is identical to garden variety osteoarthritis. The involvement of wrists and MCP joints was first pointed out by Martel and co-workers (23), and by Hamilton and colleagues (24). These last authors found that MCP joint involvement was statistically more common in those cases associated with hemochromatosis. Flexion contractures of the affected joints, usually symmetrical, especially of the wrists, elbows and knees, and valgus, or more commonly varus, deformities of the knees are frequently found. Instances of true bony ankylosis of large joints have been reported in familial cases (10). Subchondral bone cysts, similar to the "detritus" cysts of osteoarthritis, have been noted frequently in our series, even in the first few patients (2, 22), and Martel and colleagues showed a higher incidence of such cysts in pseudogout than in matched controls with urate gout or diabetes, using the MCP joint for comparison (23). In the larger bones, such cysts can (rarely) become sufficiently large to require surgery (6). Whether such cysts are secondary phenomena or imply a primary abnormality of bone in pseudogout is unresolved.

Serial roentgenologic examination of familial cases by Zitnan & Sitaj showed that the radiopaque deposits progressed in time to involve more of a given joint as well as previously unaffected joints (9). Degenerative changes also progressed in time and appeared to follow the process of crystal deposition. We have studied several cases where intra-articular fracture, arthrotomy, or other joint trauma had occurred. The joint on the traumatized side invariably showed more marked CPPD deposition than did its contralateral mate, suggesting a relationship between injury and crystal deposition. A recent report of three cases of tabetic neurotrophic knee joints in patients with polyarticular CPPD deposits suggested that these two conditions combine to produce severe joint destruction. According to this hypothesis, tabetics with CPPD deposition are more likely to develop Charcot joints (25).

CRYSTALLOGRAPHY AND PATHOLOGY

Extensive X-ray diffraction and polariscopic study of crystals harvested from the synovial fluid of these patients and from affected tissue obtained at necropsy or surgery showed the triclinic dimorph of CPPD in every instance (7, 26). The monoclinic crystal was also present frequently. A survey of pathological calcifications in visceral organs revealed no CPPD (26); such deposits were found only in articular structures. Even in patients with pseudogout, calcifications of such structures as costal cartilage, aortic plaques, and pancreas was of the apatite series. A recent well-documented report of monoclinic CPPD in the dura mater of a patient with hyperparathyroidism, gout, and pseudogout is the first account of nonarticular deposition of this crystal (27).

An extensive joint examination was accomplished in three cases studied in life that later come to necropsy and in the seven anatomical cadavers mentioned above.[2] The distribution of CPPD paralleled that seen on radiograms. Joint capsules, particularly in the large joints and the hyaline cartilages, were affected but the heaviest deposits occurred in fibrocartilaginous structures. The menisci of the knee were involved in all. Heavy deposits were often noted in intra-articular ligaments, such as the cruciates in the knee joint. Tendons, especially the Achilles tendon, may also contain CPPD deposits. It should be emphasized that the ordinary calcific tendonitis and bursitis is associated with apatite crystal deposits, not CPPD (28).

Microscopically the deposits were composed of various sized aggregates of CPPD. The diameter of these "pseudotophaceous" chalky lesions varied from 15 μm to 0.6 cm. On gross inspection alone it was difficult to distinguish these deposits from the white, chalky MSU deposits of true gout; four additional cadavers in the above study thought initially on gross inspection to have CPPD deposits turned out after polarized light microscopic analysis to have only MSU deposits. CPPD deposits were distributed diffusely in fibrocartilage, and in the mid and superficial zones of articular cartilage. The larger deposits showed a compressed fibrous stroma containing mucopolysaccharides that stained more intensely with PAS, alcian blue, and colloidal iron than did the surrounding normal cartilage matrix. These "lakes" may arise by coalescence of smaller deposits. Some crystals occurred in areas of degeneration, but others were found in cartilage with normal staining properties. The smallest, and presumably earliest, deposits were located at the lacunar margin of the chondrocytes in the midzone. This agrees with the report of Bundens et al who studied an important biopsy specimen from a patient with severe involvement in the third decade (29). Reginato and colleagues (30) and Bjoelle (31) recently reported ultrastructural studies of cartilage in hereditary pseudogout; the former workers found that the smallest crystals were in a peculiar granular matrix and not in or near chondrocyte lacunae. Bjoelle also found no association with chondrocytes and described small areas of decreased staining in crystal-free areas and speculated that these areas are the specific lesions, possibly representing a matrix defect.

Synovial biopsy material showed acute or chronic changes compatible with the concurrent clinical state of the joint. Crystals were seen in the superficial layers of the synovium by polarized light. A recent electron microscopic study by Schumacher described CPPD both within synovial cell phagolysosomes and free in the cytoplasm (32). The latter observation prompted speculation that some of the crystals were formed de novo intracellularly.

The mechanism of CPPD deposition in cartilage is unknown. CPPD, dicalcium phosphate dihydrate [$CaHPO_4 \cdot 2H_2O$ (DCDP)], and hydroxyapatite, the three crystal forms identified in human cartilage, and $CaHPO_4$ (monetite), identified in the

[2] Kowai, K., McCarty, D. J., Unpublished data.

bones and joints of a gibbon (33), are all members of the three-component system $CaO\text{-}P_2O_5 \cdot H_2O$. These compounds have been extensively studied by soil chemists, principally by Lehr, Brown and colleagues. A review of the pertinent soil chemistry literature affords some basis for speculation because, no matter what metabolic changes underlie the process, the physicochemical conditions for formation of mineral phase CPPD must be satisfied. Brown et al have grown monoclinic CPPD in vitro in dilute electrolyte at pH 6–7 and 45° C (34). These then convert slowly to the more insoluble triclinic dimorph which, as stated above, predominates in the patients with CPPD deposition disease. As it is impossible to nucleate and grow triclinic CPPD in aqueous solution except under very acidic conditions (pH 2–3), formation of the monoclinic dimorph may be the initial event in human chondrocalcinosis.

The familial case reports and the reported association with hypophosphatasia (17) suggest a genetic (enzymatic?) defect. The association of CPPD tissue deposits with metabolic conditions such as diabetes mellitus, hyperparathyroidism, and hemochromatosis suggests an acquired defect. Since all persons with these diseases don't develop CPPD deposits, one might predict either unknown differences in the metabolic diseases, or that those afflicted are heterozygous for the CPPD defect and that the second metabolic abnormality results in the phenotypic expression of the carrier state.

PYROPHOSPHATE METABOLISM

PP_i is produced by a host of biosynthetic reactions. It contains a high-energy phosphate bond and its hydrolysis releases a great amount of free energy, strongly pulling such reactions in the direction of biosynthesis (35, 36). In general, hydrolysis occurs according to the following equation: $PP_i \xrightarrow{H_2O} 2\,P_i$. A microsomal enzyme recently described independently by Stetten and co-workers (37) and by Nordlie and co-workers (38) can also transfer a phosphoryl group to a suitable receptor such as glucose, a process that would seem more economical from the bioenergetic standpoint: $PP_i + \text{glucose} \xrightarrow{H_2O} P_i + \text{glucose 6 phosphate}$. It has been calculated that approximately 40 g of PP_i is produced each day in man during the synthesis of serum albumin alone (39). Total daily production may be of the order of kilograms. The efficiency of the pyrophosphatases may be gauged by the appearance in the urine of only 1–4 mg of pyrophosphate in 24 hr (40, 41). Urinary PP_i levels were found to be elevated in hypophosphatasia by Russell, who first proposed that PP_i was a natural substrate for alkaline phosphatase (42). Urinary PP_i was not elevated in pseudogout patients (43, 44). Plasma PP_i levels are elevated in hypophosphatasia, uremia, and in certain forms of osteomalacia (45), but not in pseudogout (44, 46).

But recently Silcox and colleagues found that the PP_i concentration in blood platelets was over 800 times that of platelet-free plasma (47). As about half of platelet PP_i was released on thrombin stimulation and as the PP_i concentration in platelets with nucleotide storage defect (48) was reduced, and as platelets contain a potent neutral inorganic pyrophosphatase not released on thrombin stimulation, it appears likely that platelet PP_i is normally packaged in the same granule as the

Table 1 Inorganic pyrophosphate levels in synovial fluid reported in various studies[a]

References	Osteoarthritis	Pseudogout	Rheumatoid Arthritis	Gout	Normal
Russell et al (4)	3.5 ± 0.38 (3)[b]	24 ± 3.8 (13)	2.6 ± 0.19 (6)	5.1 (2)	-
McCarty et al (5)	5.1 ± 0.87 (5)	14.2 ± 2.7 (16)	3.5 ± 0.5 (5)	3.1 ± 0.19 (4)	-
Altman et al (7)	12.7 ± 2.1 (11)	23.9 ± 3.0 (12)	4.6 ± 0.7 (8)	13.1 ± 3.2 (7)	3.6 ± 1.4 (5)
Silcox & McCarty (52)	9.2 ± 1.04 (35)	9.8 ± 1.03 (29)	4.2 ± 0.61 (12)	4.4 ± 0.9 (5)	–

[a] All values given in μmol/l.

[b] Numbers in parentheses in disease categories refer to the number of joints studied in that group.

nucleotides. Using a newly developed assay for PP_i in platelet-free plasma, Silcox & McCarty (49) established a normal range for PP_i ($\bar{x} \pm$ SEM $= 1.8\ \mu M \pm 0.06$). The normal values were distributed about a single mode. Plasma PP_i values in rheumatoid arthritis were also distributed normally, but in osteoarthritis they were elevated significantly ($2.62\ \mu M \pm 0.12\ P < .01$), although the distribution here was unimodal. In pseudogout the distribution was bimodal, one curve was normal, and the other elevated, but the mean of the whole population was elevated significantly with respect to the normal mean ($2.54\ \mu M \pm 0.18\ P < .01$). Until something is known of the origin of plasma PP_i, its turnover rate, and the factors influencing them, it is not possible to interpret these findings.

Russell et al (44) and McCarty and colleagues (50) have recently described increases in synovial fluid PP_i levels in pseudogout, averaging 8- and 5-fold respectively, compared to fluids from patients with other types of arthritis (1.8–6.6 μmol PP_i/l). Preliminary studies, involving incubation of joint fluid and synthetic CPPD, showed an apparent solubility of about 50 μmol/l; 118 μmol/l were dissolved in a normal plasma (50). The majority of pseudogout fluids studied by Russell and colleagues, Altman and co-workers (46), and McCarty et al (50) showed PP_i levels considerably less than this, suggesting undersaturation. Albumin and magnesium ions increased the apparent solubility, whereas calcium ions decreased it (50). Adapting a recently described enzymatic method for RNA polymerase (51), Altman et al described an ultramicromethod for the measurement of PP_i (46). Higher PP_i concentrations were found in osteoarthritis joint fluids than in fluids from rheumatoid arthritic or from normal joints. We have confirmed these findings using the yeast pyrophosphatase method (52). A summary of the results of all measurements of joint fluid PP_i performed to date is given in Table 1. It is clear that nearly all joint fluids show some increase in PP_i levels with respect to plasma concentrations. The PP_i levels in actively inflamed pseudogout joints were lower ($\bar{x} \pm$ SEM $5.4 \pm 0.5\ \mu M$) than were those from chronically symptomatic joints ($10.4 \pm 1.2\ \mu M$). This is probably explained by the relatively slow rate of crystal dissolution (1–2 days)[3] relative to the rate of turnover of the articular PP_i pool (1–2 hr) (53). The level of PP_i in osteoarthritis joint fluid was positively correlated with the degree of degenerative change as assessed radiologically (52).

---

[3] Bennett, R. M., Lehr, J. R., McCarty, D. J., Unpublished data.

The source(s) of synovial fluid PP_i is unknown. The synovial cells might be expected to make large quantities of PP_i in the course of hyaluronate and protein synthesis. Chondrocytes are even better candidates as they make protein-polysaccharide and sulfate it extensively, and activation of inorganic sulfate produces PP_i (35). Howell has reported that immature rabbit cartilage, incubated in short term tissue culture, liberates PP_i into the surrounding medium, but mature rabbit cartilage does not.[4] And Mankin has recently pointed out that chondrocytes revert to a primitive chondroblastic state in osteoarthritis, with resumption of mitotic activity and increased synthesis of matrix (54). The elevated joint fluid level of PP_i in osteoarthritis might be explained by this presumed attempt at cartilage repair. Bone also contains much PP_i and it is conceivable that PP_i enters the cartilage through recently described defects in the zone of provisional calcification, and thence to joint fluid (55). In any event, it now appears that there is excess PP_i (as compared to plasma) in most joints afflicted with arthritis of nearly any type. Why nucleation as the calcium salt proceeds in some instances and not in others remains to be determined. That CPPD crystals occur in rheumatoid joints is of particular interest, because it is evident that MSU crystals never do. There is a strong negative correlation between true gout and rheumatoid arthritis!

Zitnan & Sitaj (9) have postulated a primary defect in cartilage matrix with secondary formation of a mineral phase, and the recent electron microscopic studies lend support to this idea (30, 31). We have postulated a defect in PP_i metabolism, perhaps a deficiency of an inorganic pyrophosphatase (56). The reported occurrence of pseudogout in a woman with hypophosphatasia (17) and the evidence that PP_i is a natural substrate for alkaline phosphatase (57), as Russell had predicted, suggests that a deficiency of this enzyme may be causally related to the CPPD deposition. Alkaline phosphatase and many other enzymes, both membrane-bound and soluble, have been found to have pyrophosphatase activity. Such enzymes have been described in the cytosol and in virtually all subcellular organelles including the nucleus.

Russell et al (44) and Yaron and co-workers (58) have reported decreased alkaline phosphatase activity in pseudogout joint fluid. But determination of pseudogout and control fluid enzymatic activities against two synthetic substrates (P-nitrophenylphosphate and β-glycerophosphate) showed no such differences (50). Moreover, studies of alkaline phosphatase isoenzymes in these fluids showed a similar distribution of the liver, intestinal, and bone enzyme.

A preliminary report described decreased joint fluid levels of a pyrophosphate phosphotransferase in gout and pseudogout compared with control fluids from patients with rheumatoid arthritis (59). But no differences in total inorganic pyrophosphatase activity were found in joint fluids from chronically symptomatic pseudogout when compared with osteoarthritis joint fluid, perhaps a more meaningful comparison.[5] The activities in both were very low. Total activity was partly dependent on magnesium ions and it was inhibited by other divalent cations. Nearly identical activities of a potent, soluble, neutral pyrophosphatase were found in erythrocytes from pseudogout patients and controls (56). This enzyme was markedly inhibited by divalent iron, calcium, and copper, leading to speculation that similar enzymes in articular tissues might be similarly inhibited in those cases of CPPD deposition associated with hyperthyroidism, hemochromatosis, and Wilson's disease respectively.

---

[4] Howell, D. S., Personal communication.

[5] Jacobelli, S., McCarty, D. J., Unpublished data.

The treatment of both gout (MSU crystal-induced arthritis) and hyperuricemia is specific and spectacularly successful (60). None should suffer its ravages now. The acute attack is readily aborted by oral administration of either colchicine or indomethacin. This anti-inflammatory effect may be mediated by block of chemotactic factor release from leukocytes as shown by Phelps and co-workers (reviewed in reference 61). Phenylbutazone given orally or colchicine given intravenously are probably the most potent agents for rapid reversal of gouty inflammation—the former is useful when the diagnosis is known and the response to colchicine is incomplete, and the latter is useful in treating postoperative attacks or in other clinical situations where oral medications cannot be given. Colchicine may cause serious toxicity when given to elderly patients (diarrhea with electrolyte imbalance) or to patients with depressed bone marrow activity who are taking chemotherapeutic agents. Hyperuricemia may be readily controlled in most patients with drugs such as probenecid or sulfinpyrazone, which block tubular reabsorption of urate, or by displacing protein-bound urate as suggested by Klinenberg and colleagues (reviewed in reference 62). Patients receiving chemotherapeutic agents for hematopoietic conditions, or who have formed urate stones in the past, or with poor renal function, can be satisfactorily managed with the xanthine oxidase inhibitor allopurinol. Infammatory manifestations of gout should be under satisfactory control before therapy for hyperuricemia is begun, as there is an increased risk of initiating new attacks of arthritis and of suddenly causing flare-ups in smouldering joints when the blood urate level is suddenly reduced. A modified low purine diet is advisable for most patients in order to avoid sudden rises in plasma urate. Weight reduction in an obese subject will often result in a drop in plasma urate concentrations. An augmented fluid intake is advisable for most patients taking uricosuric drugs. Mild alkalinization of the urine is often helpful, as urate solubility is considerably enhanced.

Acute or subacute attacks of pseudogout are readily recognized by identification of CPPD crystals in synovial fluid leukocytes using compensated polarized light microscopy (63). Such attacks usually respond to thorough aspiration of the joint through a 20 gauge or larger needle. Injection of microcrystalline adrenocorticosteroid esters can be accomplished at the same time and will rapidly control even the most severe attacks. When needle aspiration is not feasible or when many joints are affected, treatment with colchicine, indomethacin, or phenylbutazone is often successful, using the same regimen as in true urate gout. Interestingly, the release of chemotactic factor from neutrophils phagocytosing urate crystals in vitro is nearly always blocked by colchicine in concentrations readily obtained in vivo in man (50% or greater inhibition), whereas release of this factor by CPPD phagocytosis is blocked to this extent only in a minority of instances (64). That is, the unpredictability of colchicine effectiveness in the test tube parallels the unpredictability of the drug in treating acute pseudogout clinically.

No way has yet been devised to prevent CPPD crystal deposition in tissues or to remove such deposits once they have formed. Successful efforts to remove them at an early stage of the disease might be beneficial in reducing the number of acute and subacute attacks of inflammation, and if the crystals are causally related to the accompanying degenerative process, might also be prophylactic in this regard. As the knee is affected most commonly, we have contemplated treatment by lavage with solutions that promote CPPD solubilization.

Literature Cited

1. McCarty, D. J. 1970. *Ann. Rev. Med.* 21:357–66
2. McCarty, D. J. 1963. *Geriatrics* 18:467–78
3. Wyngaarden, J. B. 1972. *Arthritis and Allied Conditions,* ed. J. L. Hollander, D. J. McCarty, 1071–111. Philadelphia: Lea & Febiger. 8th ed.
4. Henderson, J. F. 1972. Regulation of purine biosynthesis. *ASC Monogr. 170,* ed. F. Marshall Beringer, 1–304. Washington D.C.: Am. Chem. Soc.
5. McCarty, D. J. 1972. *Arthritis and Allied Conditions,* ed. J. L. Hollander, D. J. McCarty, 1140–60. Philadelphia: Lea & Febiger. 8th ed.
6. McCarty, D. J., Silcox, D. C. 1973. *Geriatrics* 28:110–20
7. McCarty, D. J., Hogan, J. M., Gatter, R. A., Grossman, M. 1966. *J. Bone Joint Surg.* 48A:309–25
8. Katz, W. A., Schubert, M. 1970. *J. Clin. Invest.* 49:1783
9. Zitnan, D., Sitaz, S. 1966. *Acta Rheum. Baln. Pist.* 2:9–71
10. Reginato, A. M., Valenzuela, F. R., Martinez, V. C., Passano, G., Daza, S. 1970. *Arthritis Rheum.* 13:197–213
11. Geerards, J., Van der Korst, J. K. 1973. *Ann Rheum. Dis.* 32:87
12. Delbarre, F. 1964. *Presse Med.* 72:2973–78
13. deSèze, S., Hubault, A., Welfring, J., Kahn, M. F., Solonica, J. 1964. *Rev. Rhum.* 31:479–85
14. Hamilton, E., Williams, R., Barlow, K. A., Smith, P. M. 1968. *Quart. J. Med.* 37:171–82
15. Gordon, D. A., Little, H. A. 1973. *Arthritis Rheum.* 16:305–12
16. McCarty, D. J. 1973. XIII International Congress of Rheumatology, Kyoto, Japan. *Excerpta Med. Found. Int. Congr. Ser. n299,* p. 80
17. O'Duffy, J. D. 1970. *Arthritis Rheum.* 131:381–88
18. Boudin, G., Pepin, B., Hubault, A. 1963. *Bull. Soc. Med. Hop. Paris* 114:617–22
19. Schumacher, H. R. 1972. *Arthritis Rheum.* 15:259–66
20. Bywaters, E. G. L. 1972. *Ann. Rheum. Dis.* 31:219–21
21. Good, A. E., Rapp, R. 1967. *N. Engl. J. Med.* 277:286–90
22. McCarty, D. J., Kohn, N. N., Faires, J. S. 1962. *Ann. Intern. Med.* 56:711–37
23. Martel, W., Champion, C. K., Thompson, G. R., Carter, T. L. 1970. *Am. J. Roentgenol.* 109:587–605
24. Atkins, C. J., McIvor, J., Smith, P. M., Hamilton, E., Williams, R. 1970. *Quart. J. Med.* 39:71–82
25. Jacobelli, S., McCarty, D. J., Silcox, D. C., Mall, J. C. 1973. *Ann. Intern. Med.* 79:340–47
26. Gatter, R. A., McCarty, D. J. 1967. *Arch. Pathol.* 84:346–53
27. Grahame, R., Sutor, D. J., Mitchener, M. B. 1971. *Ann. Rheum. Dis.* 30:597–604
28. McCarty, D. J., Gatter, R. A. 1966. *Arthritis Rheum.* 9:804–19
29. Bundens, W. D., Brighton, C. T., Weitzman, G. 1965. *J. Bone Joint Surg.* 47A:111–22
30. Reginato, A. M., Schumacher, H. R., Martinez, V. A. 1973. *Arthritis Rheum.* 16:171–78
31. Bjoelle, A. O. 1972. *Ann. Rheum. Dis.* 31:449–56
32. Schumacher, H. R. 1968. *Arthritis Rheum.* 11:426–35
33. Murphy, R. C., Sullivan, W. E. 1958. *Med. Radiogr. Photogr.* 34:80–81
34. Brown, E. H., Lehr, J. R., Smith, J. P., Frazier, A. W. 1963. *J. Agr. Food Chem.* 11:214–22
35. Kornberg, A. 1962. *Horizons in Biochemistry, Albert Szent-Gyorgyi.* Dedicatory Volume, ed. M. Kasha, B. Pullman, 251–64. New York: Academic

36. Stetten, D. Jr. 1960. *Am. J. Med.* 28:867–70
37. Stetten, M. R., Taft, H. L. 1964. *J. Biol. Chem.* 239:4041–46
38. Arion, W. J., Nordlie, R. C. 1964. *J. Biol. Chem.* 239:2752–57
39. Fleisch, H. A., Russell, R. G. G. 1970. *International Encyclopedia of Pharmacology and Therapeutics,* ed. H. Rasmussen, Sec. 51, p. 61–329. Oxford: Pergamon
40. Fleisch, H. A., Bisaz, S. 1962. *Am. J. Physiol.* 203:671–75
41. Lewis, A. M., Thomas, W. C. Jr., Tomita, A. 1966. *Clin. Sci.* 30:389–97
42. Russell, R. G. G. 1965. *Lancet* 2:461–64
43. Pflug, M., McCarty, D. J., Kawahara, F. 1969. *Arthritis Rheum.* 12:228–31
44. Russell, R. G. G. et al 1970. *Lancet* 2:899–902
45. Russell, R. G. G., Bisaz, S., Donath, A., Morgan, D. B., Fleisch, H. A. 1971. *J. Clin. Invest.* 50:961–69
46. Altman, R. D., Muniz, O., Pita, J. C., Howell, D. S. 1973. *Arthritis Rheum.* 16:171–78
47. Silcox, D. C., Jacobelli, S. G., McCarty, D. J. 1973. *J. Clin. Invest.* 52:1595–1600
48. Holmsen, H., Weiss, H. J. 1970. *Brit. J. Haematol.* 19:643–49
49. Silcox, D. C., McCarty, D. J. 1973. *J. Clin. Invest.* 52:1863–70
50. McCarty, D. J., Solomon, S. D., Warnock, M. L., Paloyan, E. 1971. *J. Lab. Clin. Med.* 78:216–29
51. Johnson, J. C., Shanoff, M., Bass, S. T., Boezi, J. A., Hansen, R. G. 1968. *Anal. Biochem.* 26:137–45
52. Silcox, D. C., McCarty, D. J. 1973. *Arthritis Rheum.* 16:570 (Abstr.)
53. Camerlain, M., McCarty, D. J., Silcox, D. C. 1973. *J. Clin. Invest.* In press
54. Mankin, H. J., Dorfman, H., Lippiello, L., Zarins, A. 1971. *J. Bone Joint Surg.* 53A:523–37
55. Greenwald, A. S., Haynes, D. W. 1969. *J. Bone. Joint Surg.* 51B:747–53
56. McCarty, D. J., Pepe, P. F. 1972. *J. Lab. Clin. Med.* 79:277–84
57. Cox, R. P., Griffin, M. J. 1965. *Lancet* 2:1018–19
58. Yaron, M. et al 1970. *Ann. Intern. Med.* 73:751–56
59. Good, A. E., Starkweather, W. H. 1969. *Arthritis Rheum.* 12:298
60. Smyth, C. J. 1972. *Arthritis and Allied Conditions,* ed. J. L. Hollander, D. J. McCarty, 1112–39. Philadelphia: Lea & Febiger. 8th ed.
61. McCarty, D. J. 1972. *Phagocytic Mechanisms in Health and Disease.* ed. H. H. Fundenberg, R. C. Williams, 107–21. New York: Intercontinental Med. Book Corp.
62. Klinenberg, J. 1969. *Brit. Med. J.* 4:590
63. Phelps, P., Steele, A. D., McCarty, D. J. 1968. *J. Am. Med. Assoc.* 203:508–12
64. Tse, R. L., Phelps, P. 1970. *J. Lab. Clin. Med.* 76:403–15

Copyright 1974. All rights reserved

SERUM TRIIODOTHYRONINE IN MAN

❖7113

Robert D. Utiger, M.D.
Endocrine Section, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

INTRODUCTION

Triiodothyronine (T_3) was first recognized as a secretory product of the thyroid gland over 20 years ago (1). Following its isolation and synthesis, it soon became evident that T_3 was the most potent of the calorigenic thyroactive hormones, having some 3–4 times the potency of thyroxine (T_4) in terms of its ability to restore to normal the metabolic activity of patients with hypothyroidism (2, 3). In the thyroid gland, T_3 is synthesized by coupling of monoiodotyrosine and diiodotyrosine, both already in peptide linkage in thyroglobulin, in the follicular lumen. Coupling of two diiodotyrosine residues results in T_4 formation. The exact chemical mechanism of these coupling reactions is not known though recent in vitro evidence suggests that they are enhanced by the thyroid peroxidase enzyme system (4). When iodide availability is adequate, thyroglobulin contains considerably more T_4 than T_3, the ratio of T_4 to T_3 being approximately 10–20:1 (5, 6). Thyroid tissue also contains a thyroxine deiodinase, a product of whose action is T_3 (7, 8). The contribution, if any, of this pathway to thyroidal T_3 secretion is unknown. Only recently recognized is the important finding that a substantial portion of the circulating T_3 is produced from T_4 outside the thyroid gland, that is, from the extrathyroidal monodeiodination of T_4 (9–12).

Understanding of the role of T_3 in the maintenance of the euthyroid state and the production of various states of thyroid hormone excess or insufficiency has greatly increased in recent years. This has occurred primarily as a result of the development of simple and reliable methods for measurement of serum T_3 concentrations. The rapid advances in the methodology of T_3 assay have led to wide availability of serum T_3 assays, and T_3 measurements have been shown to have important diagnostic value in certain clinical situations. This review will briefly describe some of the methodologic problems of T_3 measurement and the results of studies of serum T_3 concentrations and production in various clinical states.

T_3 TRANSPORT AND PRODUCTION

As is the case for T_4, T_3 in serum exists in both bound and free forms. Previous studies had suggested that T_3 was bound only to thyroxine binding globulin (TBG) and albumin, but recent work has demonstrated binding of T_3 to thyroxine binding prealbumin (TBPA) as well (13, 14). The affinity of T_3 for these binding proteins is clearly less than that of T_4, in vitro studies indicating that T_3 has approximately 1/30 of the affinity of T_4 for TBG (15). However, the distribution of endogenous T_3 among the various binding proteins in vivo is not known with certainty. Because the affinity of T_3 for the various binding proteins is less than that of T_4, a relatively higher proportion of the total serum T_3 exists in the free or unbound form. In serum from normal subjects, the proportion of T_3 that is unbound ranges from 0.18–0.46% of the total serum T_3 concentration (12, 16–20). Since total serum T_3 concentrations average 100–150 ng/100 ml (see below), the serum free T_3 concentration is approximately 0.1–0.4 ng/100 ml (12, 17, 18, 20). In contrast, approximately 0.03% of the total serum T_4 is unbound, resulting in a free T_4 concentration of about 2 ng/100 ml (21).

The differences in the degree of binding of the two hormones are a major explanation for their differences in fractional turnover rate, volume of distribution, serum concentration, and biological activity. The fractional turnover rate of T_3 is approximately 50–70%/day whereas that of T_4 is 10%/day. Its volume of distribution is about 35–45 l, whereas that of T_4 is 10–12 l (10, 12, 17, 22). In contrast, the serum concentration of T_4 is 60–80-fold greater than that of T_3. From such data estimates of hormone production rates can be made. It appears that the total daily production rate of T_4 is about 70–90 μg, whereas that of T_3 is about 15–30 μg (12, 17, 22–24). What is less clear is the proportion of daily T_3 production that results from extrathyroidal deiodination of T_4. That this process occurs has been demonstrated by multiple techniques (9–12, 25). That it accounts for a major portion of the T_3 produced, and also a considerable fraction of the T_4 metabolized, is clear from the evidence that hypothyroid patients receiving 0.2-mg doses of T_4 have both serum T_4 and T_3 concentrations that are normal or slightly elevated and that are present in almost the same ratio as in normal subjects (25). Using a variety of techniques, different workers have estimated that extrathyroidal T_4 to T_3 conversion accounts for 40–80% of total daily T_3 production (10–12, 24).

METHODS OF T_3 MEASUREMENT

The first reasonably practical methods for the measurement of T_3 in serum were those in which T_3 and T_4 were extracted from serum, the T_3 and T_4 separated from one another by chromatography and the T_3 measured by competitive protein-binding analysis, using TBG as the T_3 binding agent (16, 26, 27). Later studies in several laboratories indicated that this methodology resulted in overestimation of the T_3 concentration in serum, as it was shown that the chromatographic systems employed did not completely separate T_3 and T_4 and that monodeiodination of T_4 to T_3 occurred during the in vitro separation procedures (28, 29). Further refinement of these procedures subsequently led to the development of methods that appeared to obviate these problems, and serum T_3 concentration results varying from 50–75% of those originally reported were found (29, 30). Other techniques applied to T_3 measurement have included gas-liquid chromatography and double-isotope derivative methodology (31, 32). While these methods may provide reliable and precise measurements of serum T_3 concentrations, they are rather cumbersome, time-consuming, and insensitive and recently have been almost universally superseded by radioimmunoassay methods.

The development of radioimmunoassay procedures for the measurement of T_3 has now been reported by many laboratories (25, 31, 33–41). These methods use anti-T_3 sera obtained by immunization with conjugates of T_3 and various proteins or thyroglobulin, which contains T_3 in peptide linkage. T_3 may be easily covalently coupled to albumin or other proteins with the use of carbodiimides. In these conjugates the T_3 is linked via the amino or carboxyl group of its alanine side chain to carboxyl or amino groups of the albumin or synthetic protein. Anti-T_3 sera prepared by immunization with these conjugates have proven to be remarkably specific. In general, the ability of T_4 to inhibit the binding of labeled T_3 to anti-T_3 serum is no more than 1%, and in many instances much less, than that of T_3. Even this degree of reactivity may reflect T_3 contamination of the T_4 preparation used since less than 0.01% T_4 cross-reactivity has been found with some anti-T_3 sera when the T_4 preparations tested were extensively purified (25, 31). Other tetraiodinated compounds generally have been found to be even less reactive than T_4. Anti-T_3 sera also react little or not at all with such compounds as monoiodotyrosine, diiodotyrosine, and thyroglobulin. T_3 analogues such as triiodothyroacetic acid and triiodopropionic acid react well with most anti-T_3 sera, generally having about 20–80% of the reactivity of T_3 itself (25, 42). There is, however, little evidence that there are appreciable quantities of these compounds in the circulation, so their reactivity in T_3 radioimmunoassays does not limit the utility of the assays.

Although it was anticipated that reactivity of T_4 with anti-T_3 antibody might render measurement of T_3 in serum difficult, this has not proven to be a problem because of the marked specificity of most anti-T_3 sera. The presence in serum of thyroid hormone binding proteins, especially TBG, has posed a problem in the assay of T_3 in serum. The presence of these T_3 binding proteins has been shown to result in artifactually increased serum T_3 levels, undoubtedly because some of the radioactively labeled T_3 used in the assay becomes bound to the binding proteins and is thus unavailable for binding to anti-T_3 antibody. Protein binding of labeled T_3 has been circumvented in several ways. Perhaps the simplest has been with the use of various competitive inhibitors of the T_3 binding protein interaction such as 8-anilinonapthalene sulfonic acid, diphenylhydantoin, sodium salicylate, tetrachlorthyronine, and thiomerosal. Secondly, T_3 has been separated from serum by adsorption to and subsequent elution from Sephadex or by extraction of serum with ethanol (37, 39). T_3 radioimmunoassays evolved using these procedures have been found to have good reliability, precision, specificity, and sufficient sensitivity to detect serum T_3 concentrations as low as 6 ng/100 ml. An excellent review of the technical aspects of T_3 assay, as well as T_3 physiology in general, has recently been presented by Larsen (43).

SERUM T_3 IN NORMAL SUBJECTS

Serum T_3 Concentrations in Normal Subjects

Serum T_3 concentrations in a large number of normal subjects, determined in this laboratory by radioimmunoassay, are shown in Figure 1. The mean value was 110 ± 23 (SD) ng/100 ml. Significant differences in serum T_3 levels were found in normal men aged 20–39, 40–59, and 60–80 years, the mean values being 112, 102, and 83 ng/100 ml (44). There were no differences in serum T_4 concentrations in these groups. No differences in serum T_3 levels were found in women in similar age

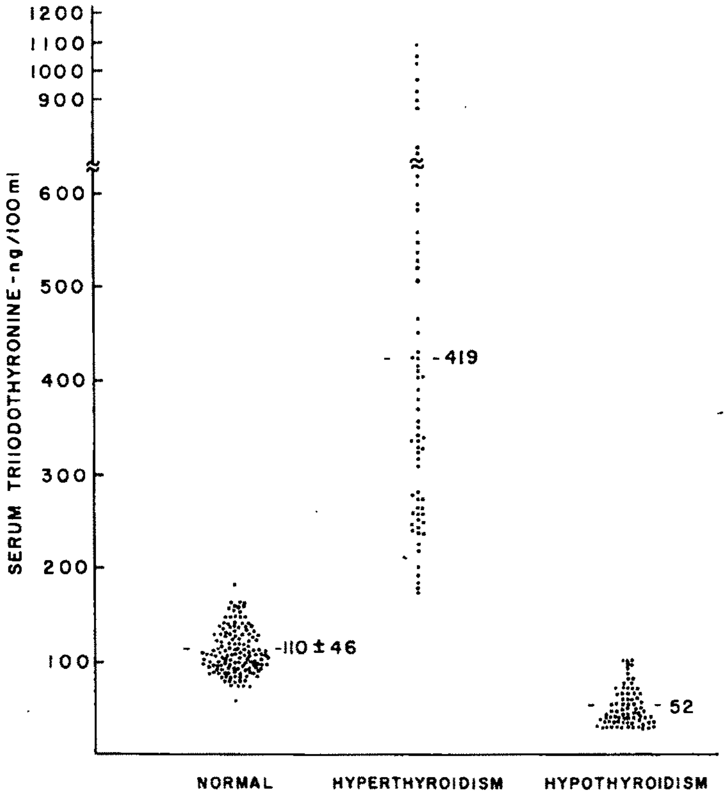


Figure 1 Serum triiodothyronine concentrations in normal subjects and patients with hyperthyroidism and hypothyroidism. Numbers indicate the mean (± two standard deviations in normal subjects).

groups (45). These results, except for the age-related decline in men, are similar to those reported from other laboratories. Thus, despite the multiplicity of technical means evolved to prevent T_3-TBG interaction in serum and the use of different anti-T_3 sera, there is general agreement that the mean serum T_3 concentrations in normal subjects range from 100–150 ng/100 ml, with one standard deviation of 20–25 ng/100 ml. In limited studies, no circadian variations in serum T_3 concentrations in normal human subjects have been found (37, 46).

TSH Effects on Serum T_3 Concentrations

Thyroidal stimulation by TSH resulting in increased serum T_3, as well as T_4, concentrations has been clearly documented by several studies. Intramuscular administration of bovine TSH (10 IU) results approximately twofold increases in both serum T_3 and T_4 concentrations (25, 36, 38). The peak increases in serum T_3 levels occur 6–8 hr after TSH administration. Peak serum T_4 concentrations generally do not occur until 24 hr after TSH administration. Serum TSH levels after such injections may reach 100–150 μU/ml (> 100-fold above normal) 2–4 hr after TSH administration (47). Increases in serum T_3 concentrations also follow increases in endogenous serum TSH concentrations induced by the intravenous administration of thyrotropin releasing hormone (TRH). Serum TSH levels increase from 2–20-fold after TRH. The peak serum TSH levels occur within 15–30 min after TRH administration and then decline to baseline levels in the ensuing 120–180 min (44, 45, 48). These increments in serum TSH result in a mean peak increase in serum T_3 concentrations of about 50%, which occurs 120–180 min after the TRH administration (38, 48, 49). Only slight, and generally insignificant, increases in serum T_4 levels occur after single intravenous doses of TRH. Thus acute transient increases in serum TSH concentrations appear to result in preferential secretion of T_3 from the thyroid gland. More sustained thyroidal stimulation results in increases in serum T_3 and T_4 levels of comparable magnitude. Though the microgram quantities of the different hormones secreted in response to the TSH can only be roughly calculated, a transient doubling of serum T_3 and T_4 concentrations would require the secretion of approximately 10 times more T_4 than T_3. More sustained increases would presumably require more nearly equal production of the two hormones since T_3 degradation is considerably more rapid than that of T_4. In the event of sustained stimulation of the normal thyroid gland by TSH, the sustained increase in T_4 secretion probably also contributes to increased T_3 production as a result of greater availability of T_4 for peripheral conversion to T_3. Thus chronic TSH stimulation should agument T_3 production in two ways.

Serum T_3 Concentrations in States of Abnormal Serum Thyroid Hormone Binding

In situations in which serum TBG (and T_4) levels are altered, parallel changes in serum T_3 concentrations have been found (19, 25, 36, 50–52). Patients who have reduced TBG levels, whether as a consequence of various diseases or of idiopathic TBG deficiency, have reduced serum T_3 as well as reduced serum T_4 concentrations. Pregnant women and patients who are receiving estrogen or who have idiopathic elevations of serum TBG all have elevated serum T_3 concentrations. The values found in such patients are generally about 50% greater than those in normal subjects. The percentage increase in serum T_3 levels that occurs in these patients is somewhat less than the increase in T_4 concentrations in the same patients.

Serum T_3 Concentrations in the Neonatal Period

Strikingly low serum T_3 concentrations have been found in cord serum obtained from newborn infants (50–52). In these subjects, T_3 levels are usually less than 50% of those in normal nonpregnant adults. Cord serum T_4 concentrations, in contrast, are similar to those found in serum from the infant's mother obtained at the time of delivery. However, serum T_3 concentrations increase rapidly following birth so that T_3 levels are well above those found in normal adults after one day of life (52). Whether the low cord serum T_3 concentrations are a result of inadequate fetal thyroidal T_3 secretion or due to reduced peripheral conversion of T_4 to T_3 in the fetus is not known. The marked rise in serum T_3 levels occurring soon after delivery is presumably a consequence of the thyroidal stimulation occurring after the marked transient increase in TSH secretion that follows birth (53). It might also reflect rapid maturation of peripheral mechanisms for T_4 conversion to T_3. The age at which normal adult serum T_3 levels are reached is not yet known.

SERUM T_3 CONCENTRATIONS IN HYPERTHYROIDISM

Serum T_3 concentrations are regularly elevated in patients with hyperthyroidism (Figure 1). In most assays, these patients are usually found to have serum T_3 levels greater than 200 ng/100 ml and the values found in groups of hyperthyroid patients average about four times the mean value found in normal subjects (25, 31, 34–41). In some instances serum T_3 levels greater than 1000 ng/100 ml have been found. In hyperthyroid patients the increase in serum T_3 concentrations is usually proportionately greater than is the increase in serum T_4 concentrations (43). The disproportionate increase in serum T_3 concentrations undoubtedly occurs as a result of both enhanced thyroidal secretion of T_3 and greater peripheral generation of T_3 due to increased T_4 availability.

The discrepancy between the increase in serum T_3 and T_4 concentrations is of the greatest magnitude in patients who have hyperthyroidism with elevated serum T_3 and normal serum T_4 concentrations ("T_3-toxicosis") (18, 54). By definition, in such patients the serum T_4 concentrations are within the normal range, although an increase in serum T_4 levels of insufficient degree to exceed the upper limit of the normal range could well have occurred. Hyperthyroidism with elevated serum T_3 and normal serum T_4 concentrations has been reported as an early manifestation of hyperthyroidism (55) in a patient who in succeeding months also develops elevated T_4 levels, as an indicator of recurrent hyperthyroidism (56), as well as in patients who have had symptomatic hyperthyroidism for long periods of time. The existence of this syndrome would appear to require two pathophysiological abnormalities. The first is any one of the disorders generally recognized as producing hyperthyroidism, such as Graves' disease or multinodular goiter. Secondly, to account for the elevation in serum T_3 concentrations alone (which presumably reflects elevation in only T_3 production rate as well), it is necessary to assume the existence of an intrathyroidal abnormality favoring T_3 production at the expense of T_4, a reduction in dietary and/or intrathyroidal iodide availability, greatly enhanced peripheral generation of T_3 from T_4, or a combination of these. No detailed studies concerning T_4 and T_3 kinetics in such patients have been reported. In North America, it has been estimated that 4% of patients with hyperthyroidism only have elevated serum T_3 concentrations (57). That reduction in iodide availability can account for this syndrome in some instances is clearly suggested by the finding that 12.5% of patients with hyperthyroidism living in Chile, an area of iodide insufficiency, had elevated serum T_3 but normal serum T_4 levels (57).

Whatever the frequency of this syndrome or its cause(s), elevated serum T_3 concentrations should be sought in any patient who has some clinical manifestations of hyperthyroidism and a normal serum T_4 concentration. Usually some palpable abnormality of thyroid size or contour will also be present. What increment in serum T_3 levels, in the absence of marked change in serum T_4 levels, is required to result in symptomatic hyperthyroidism is not known. On the basis of serum T_3 measurements in patients residing in endemic goiter regions, in patients receiving exogenous T_4 therapy, and other situations (see below), a slightly elevated serum T_3 concentration would not, by itself, be associated with significant symptomatic hyperthyroidism.

Antithyroid Therapy and Serum T_3 Concentrations

A number of different patterns of change in serum T_3 concentrations in hyperthyroid patients have been observed after administration of various antithyroid drugs. Since the turnover of T_3 is more rapid than is that of T_4, earlier reductions would be expected in serum T_3 than T_4 levels after initiation of therapy with antithyroid drugs, if blockade of thyroid hormonogenesis or release was reasonably complete. In one group of patients studied after several days of therapy with methimazole or propylthiouracil, serum T_3 levels had declined by more than 50% (58). Serum T_4 concentrations were still elevated after this treatment interval. In a group of patients treated with propylthiouracil for two weeks, serum T_3 concentrations were normal in all, but serum T_4 concentrations were still above normal in half of the patients studied (59). As yet no differences between the rate or degree of fall in serum T_3 levels which could be ascribed to a particular antithyroid drug have been found. Propylthiouracil has been known for some years to inhibit peripheral deiodination of T_4 (60) and has recently been shown to inhibit peripheral conversion of T_4 to T_3 in the rat (61) and thus might be expected to result in a more rapid reduction in serum T_3 levels than methimazole. No information is as yet available concerning early changes in serum T_3 concentrations after subtotal thyroidectomy or radioactive iodide therapy.

Serum T_3 and T_4 concentrations are both generally normal in clinically euthyroid patients who have been treated for lengthy periods with appropriate doses of antithyroid drugs or who have undergone ablative antithyroid therapy (Figure 2) (62). However, two situations in which a discrepancy between serum T_3 and T_4 levels occurs have been described in such patients. Occasionally patients have been encountered who remained hyperthyroid, or again became hyperthyroid, many weeks or months after treatment was initiated, but in whom serum T_4 levels were subnormal or normal and serum T_3 levels were elevated (62). Results of serum T_3 and T_4 assays in one such patient receiving propylthiouracil studied here are shown in Figure 3. In this patient, serum T_4 levels fell to normal but serum T_3 levels, though lower than before therapy, remained well above normal and symptomatic hyperthyroidism persisted for many months after initiation of propylthiouracil therapy. Another type of discrepancy, namely low serum T_4 and normal serum T_3 concentrations, have been found in some euthyroid patients, most of whom had previously received ^{131}I therapy (63). In all of these situations, it is reasonable to assume the presence of an intrathyroidal abnormality such that T_4 production is reduced more than T_3 production. A decrease in T_4/T_3 ratio within the thyroid gland has been demonstrated following incomplete antithyroid drug blockade of thyroid hormonogenesis in laboratory animals (64). Undoubtedly radiation damage to the thyroid gland also could result in lower intrathyroidal T_4/T_3 ratios.

SERUM T_3 CONCENTRATIONS IN HYPOTHYROIDISM

Reduced serum T_3 concentrations are usually found in patients with symptomatic hypothyroidism, whether it is due to thyroid, pituitary, or hypothalamic disease (Figure 1). Many investigators, however, have found that about 15–30% of patients with hypothyroidism, elevated serum TSH, and decreased T_4 levels have serum T_3 concentrations within the normal range (20, 25, 36, 39–40). This might simply reflect enhanced T_3 binding to TBG (and thus decreased T_3 turnover) as a direct consequence of reduced T_4 concentrations and thus increased available binding sites. More likely, the finding of normal T_3 levels in some hypothyroid patients is a reflection of both intrinsic thyroidal disease, resulting in disproportionate reduction in T_4 production, and the thyroidal effects of sustained increases in serum TSH concentrations. The net effect is that daily T_3 production is generally less depressed than is T_4 production in patients with hypothyroidism. The practical clinical point, however, is that reduced serum T_3 concentrations do not appear to be as frequent an indicator of hypothyroidism as do reduced serum T_4 or elevated serum TSH concentrations.

Serum T_3 Concentrations During Thyroid Hormone Replacement Therapy

Administration of all types of thyroid hormone preparations have been shown to result in marked changes in serum T_3 concentrations. The pattern of change that occurs depends on the type and dose of thyroid hormone treatment given. Administration of T_3 in doses of 25–100 μg daily results in prompt and striking increases in serum T_3 concentrations (20, 25, 37). The peak values generally occur 2–4 hr after T_3 administration. They range from 300–1000 ng/100 ml, depending on the T_3 dose, and return to or near the normal range by 24 hr after treatment. Clearly the mean 24-hr serum T_3 level in such patients must be well above normal, but of course their

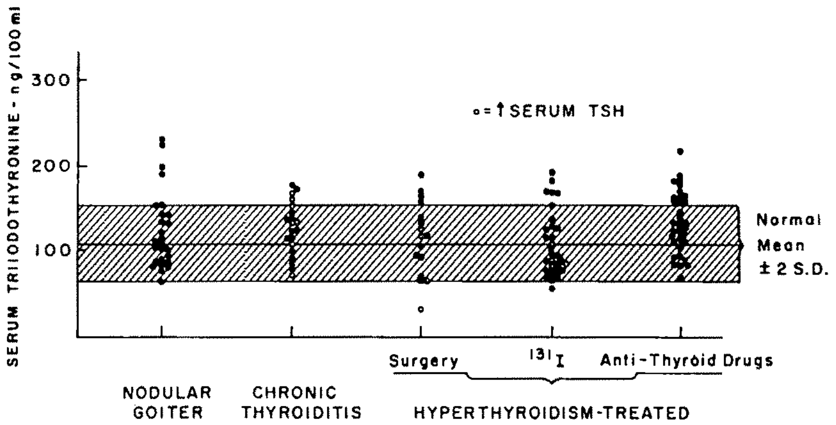


Figure 2 Serum triiodothyronine concentrations in clinically euthyroid patients with nodular goiter or chronic thyroiditis and patients with hyperthyroidism during or after various types of antithyroid therapy.

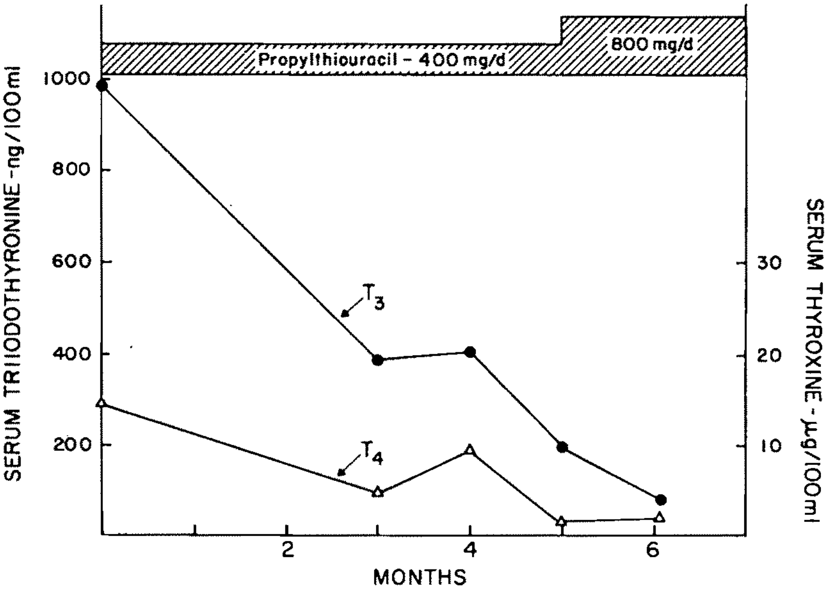


Figure 3 Serum triiodothyronine (T_3) and thyroxine (T_4) concentrations in a patient with hyperthyroidism during therapy with propylthiouracil. Note that the serum T_3 concentrations were elevated, about 400 ng/100 ml, whereas serum T_4 concentrations were normal after 3 and 4 months of therapy.

serum T_4 concentrations are subnormal at all times. Administration of mixtures of T_3 and T_4, whether given as combinations of synthetic T_3 and T_4 or as desiccated thyroid, is followed by a pattern of change in serum T_3 levels similar to that occurring after administration of T_3 alone (37, 46). The peak T_3 levels found are lower, however, since generally less T_3 is administered when it is given in combination with T_4. The practical significance of these data is that measurement of serum T_3 levels in patients receiving T_3 alone or T_3 containing thyroid preparations will yield uninterpretable results unless the exact time of hormone administration is known.

T_4 treatment also results in increases in serum T_3 concentrations but with an entirely different time pattern of change. No increase in serum T_3 levels occur within hours after T_4 administration (37), but rather very gradually, reaching a stable level only after weeks of T_4 treatment (Figure 4, *top*). As shown in Figure 4 (*bottom*), it is now generally agreed that patients receiving T_4 in doses of 0.2 or 0.3 mg daily have serum T_3 levels that are high normal or somewhat elevated. Such patients also have high normal or somewhat elevated serum T_4 concentrations. The now inescapable conclusion is that these doses of T_4 result in iatrogenic hyperthyroidism, though its degree is too small to be clinically manifest in most patients. This is corroborated by the finding that virtually all patients with hypothyroidism treated with these quantities of T_4 have serum TSH concentrations that are normal or undetectable (65, 66).

SERUM T_3 IN OTHER THYROID DISEASES

Endemic Goiter

Reductions in iodide intake are known to be associated with reductions in serum T_4 concentrations and have been clearly shown to be associated with increases in thyroidal T_3/T_4 ratio, both in man and experimental animals, leading to the supposition that elevated serum T_3 levels would be found in such populations. Recent studies in a group of subjects living in an endemic goiter region in the Himalayas have demonstrated moderately increased serum T_3 concentrations in the goitrous individuals (67). These subjects were considered to be clinically euthyroid but had elevated serum TSH concentrations. These results clearly indicate that altered availability of iodide influences the relative proportions of T_3 and T_4 produced and secreted by the thyroid gland, and also that this process may be facilitated by increased serum TSH levels. It would also appear that modestly elevated serum T_3 concentrations alone are not sufficient to fully maintain the euthyroid state since these goitrous subjects had elevated serum TSH levels, a finding now generally accepted as indicative of at least physiological, though not necessarily clinically evident, thyroid insufficiency (68).

Euthyroid Goiter

Patients who have nodular goiter or chronic thyroiditis (Hashimoto's disease) and who are considered clinically euthyroid generally have serum T_3, as well as serum T_4, concentrations that are within the normal range. Minor elevations in serum T_3

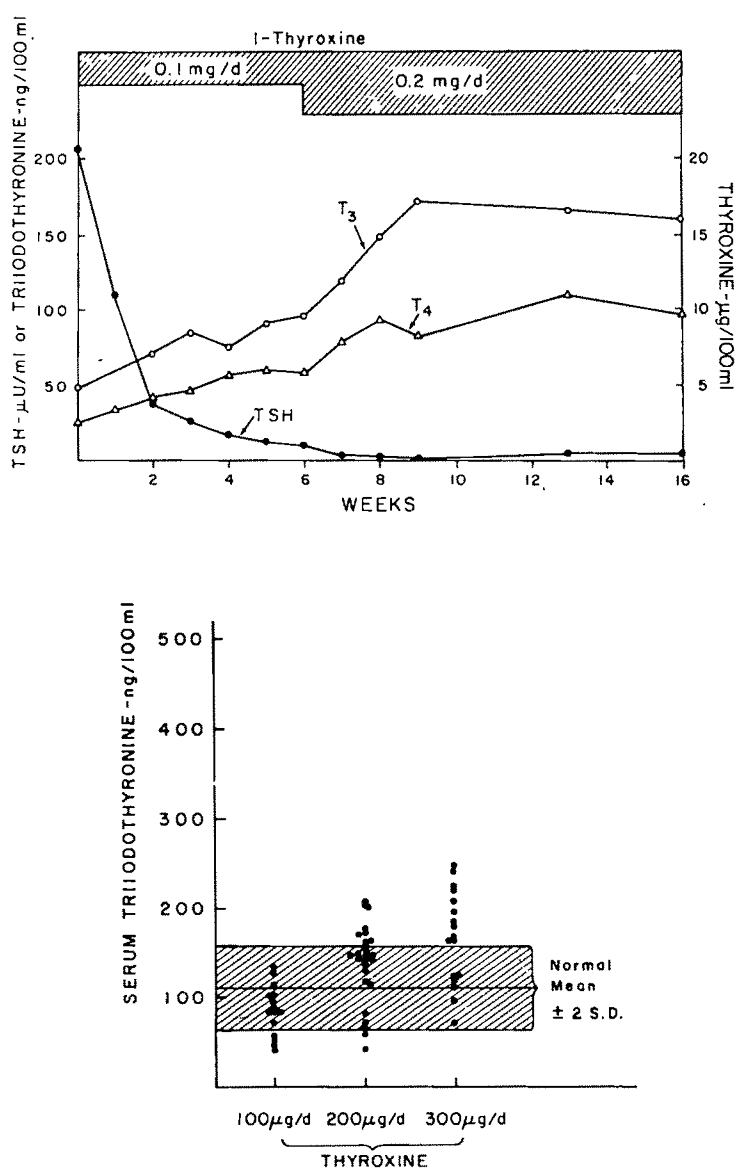


Figure 4 Top panel — results of serum TSH, triiodothyronine (T_3) and thyroxine (T_4) measurements in a patient with primary hypothyroidism during treatment with thyroxine. *Bottom panel* — serum T_3 concentrations in hypothyroid patients receiving chronic therapy with various doses of thyroxine.

concentrations are present in some patients with nodular goiter (Figure 2). Such elevations suggest that much of the thyroid tissue in such patients is functioning autonomously and may presage the development of hyperthyroidism. The serum hormone levels found in patients with chronic thyroiditis generally accurately reflect the clinical status (69). Some patients with both normal serum T_3 and T_4 levels have elevated serum TSH concentrations (Figure 2). This finding indicates that, even though the serum T_3 and T_4 levels are both within the normal range, they are probably lower than those present preceding the development of the thyroid disease. The failure of serum T_3 concentrations to increase after TRH administration in some euthyroid patients with chronic thyroiditis is a further reflection of the presence of impaired thyroid reserve capacity in these patients (49). This may also be a reflection of the finding that thyroid tissue from a small group of such patients has been found to have very low concentrations of T_3 (6).

CONCLUSION

Serum T_3 measurements have provided much of the evidence that a major proportion of T_3 production occurs at extrathyroidal sites. Investigation into the factors that regulate this process is only beginning. One outgrowth of this work is the concept that T_4 is primarily a prohormone and that it exerts biological activity only after conversion to T_3. That T_4 is a prohormone is clearly established now, but whether or not T_4 itself is incapable of exerting biological activity in tissues is not. The question is perhaps not likely to be resolved completely by in vivo studies, unless agents that prevent extrathyroidal T_4 to T_3 conversion can be found.

The ability to determine serum T_3 concentrations has also led to significant understanding of the consequences of certain types of thyroid disease and the effects of certain types of thyroid and antithyroid therapy. Such measurements have, for example, provided an explanation for the presence of hyperthyroidism in some patients and the maintainence of the euthyroid state in others. It is perhaps too early to assess the role of T_3 measurements in routine clinical medicine but certainly in some situations knowledge of the serum T_3 concentration provides a truer indication of the "thyroid status" of an individual patient than do other procedures.

ACKNOWLEDGMENT

The studies described herein carried out in this laboratory were supported in part by United States Public Health Service Grants TO1 AM05649 and R01 AM14039. The author wishes to acknowledge the expert secretarial help of Miss Rita Plume.

Literature Cited

1. Gross, J., Pitt-Rivers, R. 1952. *Lancet* 1:439–41
2. Gross, J., Pitt-Rivers, R., Trotter, W. R. 1952. *Lancet* 1:1044–45
3. Lerman, J. 1952. *J. Clin. Endocrinol. Metab.* 13:1341–46
4. Lamas, L., Dorris, M. L., Taurog, A. 1972. *Endocrinology* 90:1417–26
5. Nagataki, S., Uchimura, H., Masuyama, Y., Nakao, L., Ito, K. 1972. *J. Clin. Endocrinol. Metab.* 35:18–23
6. Chopra, I. J., Fisher, D. A., Solomon, D. H., Beall, G. N. 1973. *J. Clin. Endocrinol. Metab.* 36:311–16
7. Dawber, N. A., Galton, V. A., Ingbar, S. H. 1971. *Endocrinology* 88:144–48
8. Haibach, H. 1971. *Endocrinology* 88:918–23
9. Braverman, L. E., Ingbar, S. H., Sterling, K. 1970. *J. Clin. Invest.* 49:855–64
10. Pittman, C. S., Chambers, J. B., Read, V. H. 1971. *J. Clin. Invest.* 50:1187–96
11. Surks, M. I., Schadlow, A. R., Stock, J. M., Oppenheimer, J. H. 1973. *J. Clin. Invest.* 52:805–11
12. Braverman, L. E. et al 1973. *J. Clin. Invest.* 52:1010–17
13. Larsen, P. R. 1972. *J. Clin. Invest.* 51:1125–34
14. Davis, P. J., Handwerger, B. S., Gregerman, R. I. 1972. *J. Clin. Invest.* 51:515–21
15. Hao, Y. L., Tabachnick, M. 1971. *Endocrinology* 88:81–92
16. Nauman, J. A., Nauman, A., Werner, S. C. 1967. *J. Clin. Invest.* 46:1346–55
17. Oddie, T. H., Fisher, D. A., Dussault, J. H., Thompson, C. S. 1971. *J. Clin. Endocrinol. Metab.* 33:653–60
18. Hollander, C. S. et al 1972. *Lancet* 1:609–11
19. Dussault, J. H., Fisher, D. A., Nicoloff, J. T., Row, V. W., Volpe, R. 1973. *Acta Endocrinol.* 72:265–71
20. Chopra, I. J., Solomon, D. H., Chua Teco, G. N. 1973. *J. Clin. Endocrinol. Metab.* 36:1050–57
21. Woeber, K. A. 1971. *The Thyroid,* ed. S. Werner, S. Ingbar, 256–66. New York: Harper & Row. 914 pp.
22. Nicoloff, J. T., Low, J. C., Dussault, J. H., Fisher, D. A. 1972. *J. Clin. Invest.* 51:473–83
23. Fisher, D. A., Oddie, T. H., Thompson, C. S. 1971. *J. Clin. Endocrinol. Metab.* 33:647–52
24. Singer, P., Nicoloff, J. T. 1972. *J. Clin. Endocrinol. Metab.* 35 82–89
25. Lieblich, J., Utiger, R. D. 1972. *J. Clin. Invest.* 51:157–66
26. Sterling, K., Bellabarba, D., Newman, E. S., Brenner, M. A. 1969. *J. Clin. Invest.* 48:1150–58
27. Wahner, H. W., Gorman, C. A. 1971. *N. Engl. J. Med.* 284:225–30
28. Fisher, D. A., Dussault, J. H. 1971. *J. Clin. Endocrinol. Metab.* 32:675–79
29. Larsen, P. R. 1971. *Metabolism* 20:609–24
30. Dussault, J. H., Lam, R., Fisher, D. A. 1971. *J. Lab. Clin. Med.* 77:1039–50
31. Mitsuma, T., Nihei, N., Gershengorn, M. C., Hollander, C. H. 1971. *J. Clin. Invest.* 50:2679–88
32. Hagen, G. A., Diuguid, L. I., Kliman, B., Stanbury, J. B. 1973. *Biochem. Med.* 7:191–202
33. Brown, B. L., Ekins, R. P., Ellis, S. M., Reith, W. S. 1970. *Nature* 226:359
34. Gharib, H., Mayberry, W. E., Ryan, R. J. 1970. *J. Clin. Endocrinol. Metab.* 31:709–12
35. Chopra, I. J., Solomon, D. H., Beall, G. N. 1971. *J. Clin. Invest.* 50:2033–41
36. Larsen, P. R. 1972. *J. Clin. Invest.* 51:1939–49
37. Surks, M. I., Schadlow, A. R., Oppenheimer, J. H. 1972. *J. Clin. Invest.* 51:3104–13
38. Chopra, I. J., Ho, R. S., Lam, R. 1972. *J. Lab. Clin. Med.* 80:729–39
39. Patel, Y. C., Burger, H. C. 1973. *J. Clin. Endocrinol. Metab.* 36:187–90
40. Hufner, M., Hesch, R. D. 1973. *Acta Endocrinol.* 72:464–74
41. Hesch, R. D., Evered, D. 1973. *Brit. Med. J.* 1:645–48
42. Gharib, H., Ryan, R. J., Mayberry, W. E., Hockart, T. 1971. *J. Clin. Endocrinol. Metab.* 33:509–16
43. Larsen, P. R. 1972. *Metabolism* 21:1073–92
44. Snyder, P. J., Utiger, R. D. 1972. *J. Clin. Endocrinol. Metab.* 34:380–85
45. Snyder, P. J., Utiger, R. D. 1972. *J. Clin. Endocrinol. Metab.* 34:1096–98
46. Snyder, P. J., Utiger, R. D. 1972. *J. Clin. Invest.* 51:2077—84
47. Hershman, J., Edwards, C. L. 1972. *J. Clin. Endocrinol. Metab.* 34:814–18
48. Shenkman, L., Mitsuma, T., Suphavai, A., Hollander, C. S. 1972. *Lancet* 1:111–13
49. Shenkman, L., Mitsuma, T., Hollander, C. S. 1973. *J. Clin. Endocrinol. Metab.* 36:1074–78

50. Lieblich, J. M., Utiger, R. D. 1973. *J. Pediat.* 82:290–92
51. Fisher, D. A., Dussault, J. H., Hobel, C. J., Lam, R. 1973. *J. Clin. Endocrinol. Metab.* 36:397–400
52. Abuid, J., Stinson, D. A., Larsen, P. R. 1973. *J. Clin. Invest.* 52:1195–99
53. Fisher, D. A., Odell, W. D. 1969. *J. Clin. Invest.* 48:1670–77
54. Sterling, K., Refetoff, S., Selenkow, H. 1970. *J. Am. Med. Assoc.* 213:571–75
55. Hollander, C. S. et al 1971. *Lancet* 2:731–33
56. Shenkman, L., Mitsuma, T., Blum, M., Hollander, C. S. 1972. *Ann. Intern. Med.* 77:410–13
57. Hollander, C. S. et al 1972. *Lancet* 2:1276–78
58. Larsen, P. R. 1972. *Clin. Res.* 20:431
59. Shenkman, L. S., Mitsuma, T., Hollander, C. S. 1973. *J. Clin. Invest.* 52:205–9
60. Morreale de Escobar, G., Escobar del Ray, R. 1967. *Recent Progr. Horm. Res.* 23:87–131
61. Oppenheimer, J. H., Schwartz, H. L., Surks, M. I. 1972. *J. Clin. Invest.* 51:2493–97
62. Hollander, C. S., Shenkman, L., Mitsuma, T., Asper, S. P. 1972. *Johns Hopkins Med. J.* 131:184–88
63. Sterling, K., Brenner, M. A., Newman, E. S., Odell, W. H., Bellabarba, D. 1971. *J. Clin. Endocrinol. Metab.* 33:729–31
64. Richards, J. B., Ingbar, S. H. 1959. *Endocrinology* 65:198–207
65. Utiger, R. D. 1970. *Proceedings of the Midwest Conference on the Thyroid,* ed. A. Kenny, R. Anderson, 64–85. Columbia, Mo.: Univ. Mo. 133 pp.
66. Cotton, G. E., Gorman, C. A., Mayberry, W. E. 1971. *N. Engl. J. Med.* 285:529–33
67. Kochupillai, N. et al 1973. *Lancet* 1:1021–24
68. Utiger, R. D. 1971. *Ann. Intern. Med.* 74:627–29
69. Gharib, H., Wahner, H. W., McConahey, W. M. 1972. *Mayo Clin. Proc.* 47:175–79

Copyright 1974. All rights reserved

MOLECULAR IMMUNOLOGY OF FACTOR VIII[1]

❖7114

Theodore S. Zimmerman, M.D.[2] and Thomas S. Edgington, M.D.
Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California

Molecular immunology has served to better characterize the molecular defects underlying the two principal inherited abnormalities of Factor VIII—classic hemophilia and von Willebrand's disease. Of particular significance has been the ability of specific antibodies to detect Factor VIII molecules that are partially or totally deficient in their procoagulant activity. Such antibodies have served as molecular probes to establish the presence of a functionally defective Factor VIII molecule in classic hemophilia, in contradistinction to the true quantitative deficiency of the Factor VIII molecule in von Willebrand's disease. Appropriately characterized antibodies may serve as biological probes of fine molecular structure and can be utilized to discriminate between various intramolecular lesions. Thus it has been possible to identify one form of hemophilia in which absence of Factor VIII procoagulant activity is nevertheless accompanied by retention of antigenic determinants apparently associated with the functional site. This contrasts with a second, more common, and usually more severe form of hemophilia, in which these functional site-associated antigenic expressions are lost or modified in concert with the molecular change(s) associated with this form of functionally defective Factor VIII molecule.

The application of serologic and more sophisticated immunochemical techniques has identified a molecule that is decreased or absent from plasma in von Willebrand's disease. This molecule, variably referred to as Factor VIII-like antigen or von Willebrand's disease antigen, is present in hemophilia in quantities comparable to that found in normal plasma. This von Willebrand's disease antigen, although biologically linked to Factor VIII in an as yet unestablished fashion, nevertheless appears to be distinct from the molecule possessing Factor VIII procoagulant

---

[1] This is publication number 742 of the Department of Experimental Pathology, Scripps Clinic and Research Foundation. Supported by NIH Research Grants HL-15491 and HL-15216 and Training Grant 5-TO1-GM-00683-12.

[2] Recipient of NIH Career Development Award IKO4 HL 70242.

Table 1 Definition of terms

Factor VIII Procoagulant Activity (Antihemophilic Factor, AHF): An activity which corrects the coagulation abnormality of hemophilic blood or plasma. Measured by coagulation assays. Decreased in both classic hemophilia and von Willebrand's disease. Probably resides on a molecule distinct from that expressing the von Willebrand's antigen.

The von Willebrand's Disease Factor: An activity which confers glass bead adhesiveness and ristocetin aggregatability on human platelets. Decreased in most cases of von Willebrand's disease, normal in classic hemophilia. Its lack may be responsible for the prolonged bleeding time in von Willebrand's disease. Biologically linked to Factor VIII and the von Willebrand's disease antigen.

The von Willebrand's Disease Antigen (AHF-like antigen, Factor VIII-like antigen, vW-Ag): A precipitating antigen which is decreased or absent in most cases of von Willebrand's disease, normal in classic hemophilia.

Hemophilia A+ (CRM+): That subgroup of classic hemophilia in which the partially inactive Factor VIII molecule retains functional site antigenic determinants recognized by human antibodies to Factor VIII, as well as parafunctional and structural determinants recognized by rabbit antibodies to Factor VIII. In hemophilia A– only the latter antigenic determinants are retained on the inactive Factor VIII molecule.

activity. In a third frame of reference, immunologic approaches have also aided in the definition of von Willebrand's factor, an activity that also shows biologic linkage to Factor VIII and the von Willebrand's disease antigen (see Table 1).

CLASSIC HEMOPHILIA: A DEFECTIVE FACTOR VIII MOLECULE

Our current understanding of the molecular defect underlying classic hemophilia stems from observations, made originally in 1893, of a prolonged blood clotting time in this disorder (1). Later investigations established the absence from hemophilic plasmas of a clot-promoting activity present in normal plasma (2–6), and set in motion the debate as to whether this defect was attributable to the deficiency of a clotting factor, as in von Willebrand's disease, or to the presence of functionally defective clotting factor, as in hemophilia.

This problem has been explored with both human isoantibodies to Factor VIII as well as heterologous antiserum produced by immunization with Factor VIII-rich preparations. Two basic approaches have been employed. The first examines neutralization of the procoagulant activity of Factor VIII by antibodies, and is performed in a fluid phase system. Such neutralization strongly implies binding of the antibodies to antigenic determinants within or sterically proximate to the Factor VIII procoagulant site. This immunologic approach can be carried further in a two-step assay to permit detection of functionally defective Factor VIII. The first step involves preincubation of a specimen (i.e. plasma) containing defective Factor VIII with standardized antibody. The defective Factor VIII blocks the capacity of the antibody to subsequently neutralize normal Factor VIII in the second step of the fluid phase assay. The first of such experiments, performed by Shanberge & Gore in 1957 (7), did indeed show that hemophilic plasma could partially block the Factor VIII neutralizing capacity of a heterologous antiserum, suggesting the presence of a defective molecule. More extensive studies have since yielded information which, though initially contradictory, now appears to support these original conclusions. Several investigators utilizing heterologous antiserum either were not able to demonstrate these Factor VIII antigens in hemophilic plasma (8–10) or could find them only in a small minority of cases, usually those retaining some procoagulant activity (11, 12). Additional studies with antibodies having specificity for apparently different intramolecular sites have demonstrated antigenic expressions of Factor VIII in almost all hemophilic plasmas, including those without detectable Factor VIII procoagulant activity and lacking functional site-associated antigens (13–15).

More recent studies of Feinstein, Rapaport & Chong (16) suggest that the discrepant results with heterologous antisera may be partially due to low concentrations of defective Factor VIII in most hemophilics or low sensitivity of the antisera for this more defective form of the Factor VIII molecule. Utilizing a rabbit antiserum they could demonstrate normal quantities of Factor VIII antigen in only a small proportion of hemophilic plasmas or cryoprecipitates; however, Factor VIII antigens, in reduced amounts, could be detected in all hemophilic plasmas. A prior study using cryoethanol concentrates and rabbit antiserum had also demonstrated Factor VIII antigen in all severely affected hemophilics (17).

Human antibodies, capable of neutralizing Factor VIII procoagulant activity in fluid phase, provide molecular probes that give more consistent results but, because of a high degree of intramolecular specificity, do not settle the question. Approximately 10% of unrelated hemophilics have normal amounts of Factor VIII antigen detectable by these antibodies (18–22). Again, the plasmas with these Factor VIII antigens usually retain some Factor VIII procoagulant activity (approximately 3–10% of normal). These observations suggest that the human antibodies may be specific for the functional site of the Factor VIII molecule. This form of hemophilia has been designated hemophilia A+ or CRM+. As will be discussed in more detail later, it now appears that this variant of hemophilia reflects the production of a minimally defective Factor VIII molecule which retains most, if not all, of the antigenic determinants associated with the functional site of the normal Factor VIII molecule, although retaining only a small proportion of the procoagulant activity. This is in contrast to the usually more severe form of the disease in which the Factor VIII molecules appear to lack these functional site-associated determinants.

A second approach, utilizing immobilized heterologous antibodies in a primary binding system, is independent of the regional localization of antigenic determinants on the Factor VIII molecule and leaves little doubt that antigenic expressions of the Factor VIII molecule can be demonstrated in the plasma of hemophilics of all degrees of severity. Since fluid phase assays can detect antigenic determinants only within or proximate to the functional site of Factor VIII, only antibodies capable of hindering the functional sites of Factor VIII can be used. In the solid phase system antigenic determinants residing anywhere on the Factor VIII molecule, including those structural determinants remote from the functional site, can be detected. These latter studies employ a gamma globulin fraction of the rabbit anti-Factor VIII antiserum which has been covalently coupled to agarose beads. These "antibody beads" are then incubated with normal plasma to permit binding of Factor VIII to the beads. When the beads are subsequently removed by centrifugation, they remove Factor VIII molecules bound by any antigenic determinants, not simply those near the functional site. The determination of free, and therefore bound, antigen is an essential part of the binding assays, and is accomplished by assay of the supernatant plasma for residual Factor VIII coagulant activity. Evidence that binding is indeed to structural portions of the molecule follows from observations that most of the Factor VIII bound to the beads is still active in a specific Factor VIII coagulation assay, suggesting that the functional site is not bound by antibody. This system can be extended through sequential competitive inhibition to permit qualitative and quantitative immunochemical assay of Factor VIII. In such a system defective Factor VIII is detected by its ability to prevent the subsequent binding of fresh Factor VIII by the "antibody beads." Utilizing this assay, Factor VIII antigen has been detected in each of sixteen hemophilic plasmas, although the total Factor VIII antigenic expression averaged less than half that of normal plasmas (23).

Antigenic determinants of the defective Factor VIII molecule in hemophilic plasmas are sufficiently similar to those of normal Factor VIII molecule to permit exchange and competitive displacement of normal Factor VIII from immobilized antibodies by hemophilic Factor VIII. Thus, incubating antibody beads with normal plasma and washing and placing them in hemophilic plasma is followed by a significant increase in Factor VIII activity in the hemophilic plasma. In contrast, no such rise is seen if such beads are placed in the plasma of an individual with severe von Willebrand's disease (23). As will be discussed later, there is ample evidence that no defective Factor VIII is present in von Willebrand's disease plasma.

THE FACTOR VIII MOLECULE: THREE ANTIGENIC REGIONS

The superficially incompatible results obtained with human and heterologous antibody in fluid phase and rabbit antibody in solid phase immunochemical studies of Factor VIII are not unexpected if the regional segregation of antigenic determinants within molecules is accepted. A regional hypothesis for the structure and molecular immunology of Factor VIII readily accommodates currently available data. The Factor VIII molecule can be schematically considered as divisible into three antigenic regions with the procoagulant functional site as the point of reference (Figure 1, Table 2).

The first, or functional, site region (I) can be conceived of as identical to or intimately associated with the procoagulant functional site. This region is present on all normal Factor VIII molecules and also on those hemophilic Factor VIII molecules (A+, CRM+) that block human anti-Factor VIII antibody in fluid phase assay. Hemophilic Factor VIII molecules that retain the antigenic expressions of this region usually also retain limited Factor VIII procoagulant activity.

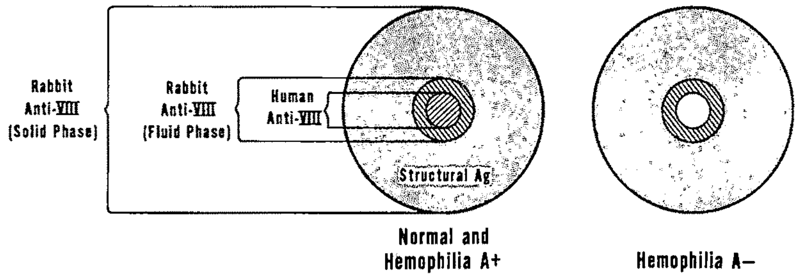


Figure 1 A hypothetical representation of antigenic regions of the Factor VIII molecule. The central area (I) represents the functional site and associated antigenic determinants detected by human antibodies to Factor VIII. This region is present on all normal Factor VIII molecules and those hemophilic molecules designated A+ or CRM+. The middle zone (II) denotes a region contiguous to the first with different antigenic determinants. Because of proximity to the functional site, binding of antibody sterically hinders the functional site. This region is detected by rabbit antibodies in fluid phase assays. The peripheral region (III) represents the remainder of the molecule and associated antigenic determinants detectable by solid phase binding techniques with rabbit antibodies. The latter two regions are present on most, if not all, hemophilic Factor VIII molecules as well as normal Factor VIII (from Zimmerman, T.S., de la Pointe, L., Edgington, T.S., Submitted for publication; with permission of the authors).

Table 2 Characteristics of proposed antigenic regions of the Factor VIII molecule

Region[a]		Steric relationship to functional site	Procoagulant activity neutralized if antibody binds to this region of normal Factor VIII molecule	Factor VIII variants in which these regions have been identified		
					Hemophiliac	
				Normal	A+ CRM+	A- CRM-
I	Functional	Intimate association, sterically identical	+	+	+	0
II	Parafunctional	Close association, sterically proximate	+	+	+	+
III	Structural	Unassociated, sterically remote	0	+	+	+

[a] Regions I and II are identified by their ability to block the Factor VIII neutralizing capacity of human (region I) or heterologous (region I & II) anti-Factor VIII antibody in fluid phase. Region III is identified in a solid phase system (see text).

A second or parafunctional region (II), which appears to be quite close to the first region, can be recognized by heterologous (rabbit) antibodies to Factor VIII which, like human antibodies, neutralize Factor VIII procoagulant activity in fluid phase. The existence of this antigenic region is established by the capacity of A– hemophilic Factor VIII to block the fluid phase neutralizing capacity of these heterologous antibodies but not that of human antibodies. A+ hemophilic Factor VIII and normal Factor VIII also block these antibodies. The presence of this region on most, if not all, hemophilic molecules—rather than just those that retain some coagulant activity—suggest that it is not intrinsic to the functional site. However, the capacity of antibodies reactive with this region to also neutralize Factor VIII indicates that region II is sterically proximate to the procoagulant site.

The third or structural antigenic region (III) can be detected with immobilized antibodies that do not neutralize Factor VIII procoagulant activity but do bind and remove the Factor VIII molecule from solution. By definition and experimental demonstration, antibodies binding to determinants in region III do not neutralize procoagulant activity, and are not detectable in fluid phase assays.

The immunochemical demonstration of functionally defective Factor VIII in fluid phase with a given antiserum will depend on its relative content of antibodies that are specific for the two antigenic regions (I and II) close to the functional site. Human antibodies appear to be specific probes for region I and do not detect region II. Heterologous antibodies may detect both, as demonstrated by Feinstein, Rapaport & Chong (16). Their heterologous antiserum detected normal quantities of Factor VIII antigen in those plasmas or cryoprecipitates in which Factor VIII antigen was detected by human antibody (i.e. A+). However, A– plasmas and cryoprecipitates exhibited incomplete (0.2–0.5 units) blocking of this heterologous anti-Factor VIII antiserum as compared with normal. These observations can be explained by a high neutralizing efficiency and thus low required concentration of antibodies to the A+ region in this antiserum, and low neutralizing efficiency with higher concentration of antibodies to the second antigenic region. Zimmerman, Ratnoff & Powell, using cryoethanol precipitates, found approximately the same Factor VIII antigenic content in hemophilics and normal plasma concentrates (17). Their antisera may have been more selective for region II. Human antibodies to Factor VIII do not detect Factor VIII antigen in A– plasma or concentrates, a property shared by the heterologous antiserum of Meyer & Larrieu (12). Thus, some heterologous antiserum appears to be as specific for region I as human antibodies, whereas others appear to exhibit specificity for region II and undoubtedly region III.

VON WILLEBRAND'S DISEASE: A TRUE DEFICIENCY OF FACTOR VIII

Von Willebrand's disease, like classic hemophilia, is a hemorrhagic disease associated with decreased Factor VIII coagulant activity of plasma. However, many features distinguish these two diseases (see Table 3). Of particular note is the response to infusions of normal plasma or Factor VIII concentrates, which produce an immediate rise in Factor VIII coagulant activity in the plasma of the hemophilic, an activity with a half-life of 12–14 hr. In contrast, infusion of Factor VIII concentrates or plasma into individuals with von Willebrand's disease is followed by a gradual rise in Factor VIII coagulant activity, sometimes not reaching a maximum for 24 hr and remaining at an increased level for as long as five days (24–27). In addition, the infusion of hemophilic plasma—containing no Factor VIII coagulant activity—is followed by a rise of Factor VIII coagulant activity in the plasma of individuals with von Willebrand's disease, although the reverse is not true. These observations suggest that the molecular abnormalities leading to a decreased Factor VIII coagulant activity are probably different in the two diseases. Immunologic studies bear this out. Techniques capable of detecting all three of the Factor VIII antigenic regions concur in showing either decreased or absent Factor VIII antigen in von Willebrand's disease. These studies include fluid phase neutralization assays with human (12, 18–20) and rabbit antibodies (17) as well as solid phase binding assays with rabbit antibodies (23). Using human antibody, a direct correlation between the reduced Factor VIII antigen and reduced Factor VIII coagulant activity is observed (12). This correlation is not seen in A+ hemophilics, whose plasma contains normal quantities of antigen as determined by human antibody, but greatly reduced Factor VIII coagulant activity. It appears that the reduced Factor VIII activity in von Willebrand's disease probably represents decreased amounts of functionally normal Factor VIII molecule. In hemophilia, on the other hand, a functionally deficient molecule, present in quantities comparable to the normal molecule, is probably responsible for the decreased coagulant activity.

THE VON WILLEBRAND'S DISEASE ANTIGEN (FACTOR VIII-LIKE ANTIGEN)

Currently used methods for the partial purification of Factor VIII frequently employ molecular exclusion through agarose columns (28–31). The exclusion volume,

Table 3 Differences and similarities between classic hemophilia and von Willebrand's disease

	Classic Hemophilia	von Willebrand's Disease
Clinical expression	Often severe with deep tissue hemorrhage; joint deformities common	Often mild; epistaxis common; joint deformities uncommon
Inheritance	Sex-linked recessive	Autosomal dominant
Bleeding time	Normal	Prolonged
Factor VIII coagulant activity (measured by coagulation assays)	Decreased	Usually decreased
von Willebrand's disease factor (measured by glass bead platelet retention, ristocetin platelet aggregation)	Normal	Usually decreased
von Willebrand's disease antigen (Factor VIII-like antigen)	Normal	Usually decreased
Response to infusion of normal plasma or Factor VIII concentrates	Maximum rise in Factor VIII activity is immediate and is accounted for by amount of Factor VIII infused.	Maximum rise in Factor VIII coagulant activity is delayed and is greater than accounted for by amount of Factor VIII infused
Development of antibodies to Factor VIII	Occurs in 5-21% of individuals	Extremely rare, if ever

containing proteins with molecular weight in the vicinity of two million or greater, is also highly enriched in Factor VIII. Antibodies prepared against such fractions in rabbits may or may not neutralize Factor VIII in fluid phase but invariably precipitate with an antigen that is often decreased or absent in plasmas of individuals with von Willebrand's disease. This antigen, designated the von Willebrand's disease (Factor VIII-like, AHF-like) antigen (see Table 1), shows a direct correlation with Factor VIII activity in the plasmas of most normal individuals (Figure 2). Like the defective Factor VIII molecule, it is present in hemophilic plasmas (17, 32–35).

Bennett & Huehns (15), utilizing a fluid phase immunoassay with rabbit antibody, have found that obligate women carriers of hemophilia often have considerably more Factor VIII antigen in their plasmas for a given amount of Factor VIII coagulant activity than normal women. Similarly, carriers of hemophilia show greater amounts of von Willebrand's antigen per unit of Factor VIII coagulant activity than normal women (36, 37). Some investigators have succeeded in identifying over 90% of carriers by this latter technique and have suggested it may be

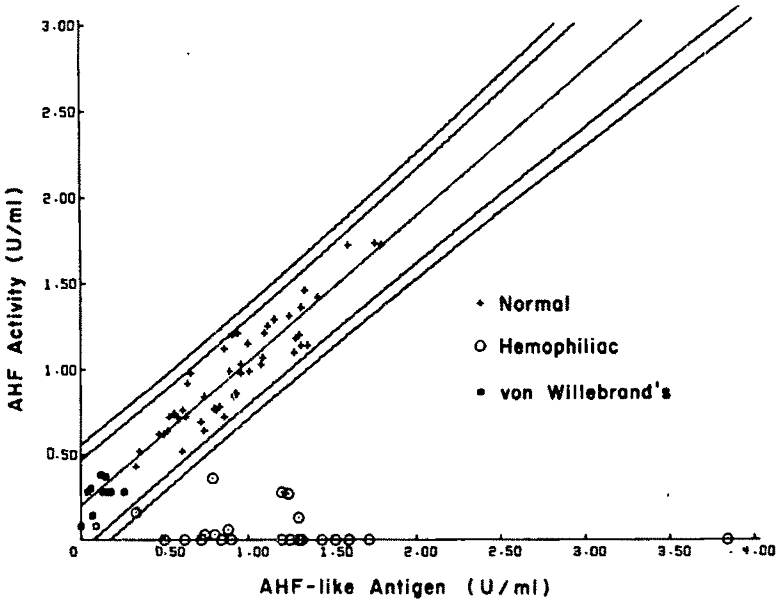


Figure 2 The relationship of AHF (Factor VIII) activity to AHF-like antigen (von Willebrand's disease antigen) in normal individuals, hemophilics, and individuals with von Willebrand's disease. The center line is the regression line for normal individuals, the outermost lines represent the 99% confidence belt, and the inner two lines the 95% confidence belt (from Zimmerman, T.S., Ratnoff, O.D., Powell, A.E. 1971. *J. Clin. Invest.* 50:244; with permission of the authors).

helpful in identifying the carrier state (36, 37). In preliminary studies, others have been able to identify only about 80% or less of carriers (38, 38a). Additional studies and improved technical precision are necessary to define the ultimate usefulness of this empirical approach.

The correlation of the von Willebrand's disease antigen with Factor VIII activity in normal individuals, and the co-isolation of von Willebrand's disease antigen and Factor VIII by most currently available techniques (28, 29, 31), indicate a close biologic linkage between these two entities, and also suggest that they may reside on the same molecule. Other observations detract from a one-molecule hypothesis. In several patients with von Willebrand's disease, Factor VIII coagulant activity has been four to six times higher than the level of von Willebrand's disease antigen (42). It also has been shown that infusion of fresh frozen plasma or cryoprecipitates into patients with von Willebrand's disease is followed by marked disparity between Factor VIII coagulant activity and the von Willebrand's disease antigen. The rise in level of the von Willebrand's disease antigen was compatible with the quantity of antigen present in the infused plasma or cryoprecipitate and declined rapidly, reaching pre-infusion of values in approximately 24 hr. However, Factor VIII procoagulant activity remained elevated and sometimes continued to rise for longer periods of time, reaching levels considerably greater than that accounted for by the amount infused (26, 27).

Direct evidence that the molecule possessing Factor VIII coagulant activity and that expressing the von Willebrand's disease antigen reside on independent molecules in plasma has been provided by primary binding studies with immobilized antibodies (39). Rabbit "antibody beads," prepared from antiserum exhibiting both the capacity to neutralize Factor VIII coagulant activity and precipitate the von Willebrand's disease antigen, removed both Factor VIII coagulant activity and von Willebrand's disease antigen from plasma. However, a greater percentage of von Willebrand's disease antigen was bound and removed than Factor VIII coagulant activity. This finding is inconsistent with a unitary hypothesis since such a hypothesis would require that the ratio of Factor VIII coagulant activity to the von Willebrand's antigen remain constant. However, the observed preferential removal of the von Willebrand's disease antigen caused this ratio to increase as increasing amounts of both are removed. The properties of antibody beads prepared with human anti-Factor VIII are also inconsistent with a unitary hypothesis. Whereas such beads removed between 52 and 64% of Factor VIII coagulant activity from normal human plasmas, they did not remove a significant amount of von Willebrand's disease antigen (see Figure 3).

THE VON WILLEBRAND'S DISEASE FACTOR

Recently, an additional factor with biologic linkage to the von Willebrand's disease antigen and Factor VIII has been described. This factor, designated the von Willebrand's factor (see Table 1), confers glass bead adhesiveness and ristocetin aggregatability on human platelets (35, 40, 41). Deficiency of this factor appears to be associated with the prolonged bleeding time characteristic of von Willebrand's disease. Like the von Willebrand's disease antigen, the factor also is present in hemophilia and often decreased in von Willebrand's disease. As is the case with Factor VIII coagulant activity, the von Willebrand's disease antigen and the von Willebrand's disease factor are isolated together under a variety of conditions (35, 39). Heterologous antisera, raised against preparations highly enriched in Factor VIII and the von Willebrand's disease antigen, neutralize the von Willebrand's factor as well as Factor VIII coagulant activity, and precipitate the von Willebrand's disease antigen (35, 42). These considerations suggest that perhaps two of these three entities may reside on a common molecule; however, attractive as it may appear, this hypothesis remains to be established.

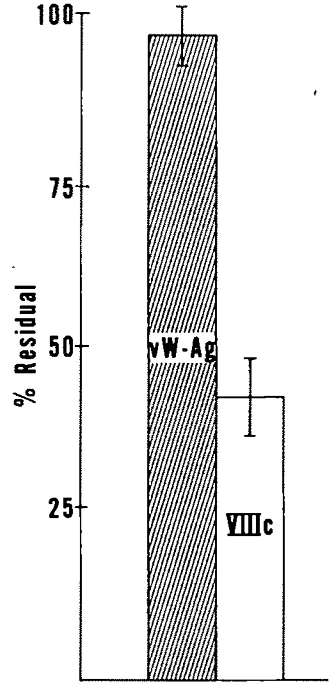


Figure 3 Removal of Factor VIII coagulant activity (VIII$_c$) from plasma, independent of von Willebrand's disease antigen (vW-Ag), by insolubilized human antibodies to Factor VIII. Human antibodies to Factor VIII were coupled to agarose beads, incubated with normal human plasma, and removed by centrifugation. The supernatant plasma was assayed for residual Factor VIII coagulant activity and von Willebrand's disease antigen. The brackets include the mean and one standard deviation for 11 determinations (from Zimmerman, T.S., Edgington, T.S. 1973. *J. Exp. Med.* 138:1015).

CONCLUSION

The use of antibodies as molecular probes has allowed the recognition of the Factor VIII molecule independent of its procoagulant activity and permitted the delineation of three antigenic regions on one molecule. This approach has established the existence of at least two forms of classical hemophilia, based on differences in the antigenic expression of the inactive Factor VIII molecule. Questions as to the relationship of the Factor VIII molecule to the molecule bearing the von Willebrand's disease antigen have been raised. The nature of this biologic linkage is open to a variety of hypothetical speculations, including a possibility that the two molecules exist in plasma as a reversible complex; the von Willebrand's molecule may be the precursor of Factor VIII or the von Willebrand's molecule may govern synthesis or release of Factor VIII from tissue sites. Elucidation of the biologic linkage of these two molecules (and their relationship to the von Willebrand's disease factor) presents an investigative challenge and should provide a sounder molecular approach to the pathobiology of those diseases associated with decreased or defective Factor VIII.

Literature Cited

1. Wright, A. E. 1893. *Brit. Med. J.* 2:223
2. Addis, T. 1911. *J. Pathol. Bacteriol.* 15: 427
3. Frank, E., Hartmann, C. 1927. *Klin. Wochenschr.* 6:435
4. Govaerts, P., Gratia, A. 1931. *Rev. Belg. Soc. Med.* 3:689
5. Bendien, W. M., Van Creveld, S. 1935. *Acta Brevia Neer.* 5:135
6. Patek, A. J. Jr., Stetson, R. J. 1936. *J. Clin. Invest.* 15:531–42
7. Shanberge, J. N., Gore, I. 1957. *J. Lab. Clin. Med.* 50:954
8. Adelson, E., Rheingold, J. J., Parker, O., Steiner, M., Kirby, J. D. 1963. *J. Clin. Invest.* 42:1040–47
9. Denson, K. W. E. 1967. *The Use of Antibodies in the Study of Blood Coagulation.* Philadelphia: Davis
10. Uszynski, L. 1967. *Thromb. Diath. Haemorrh.* 18:325–31
11. Gralnick, H. R., Abrell, E., Bagley, J. 1971. *Nature (New Biol.)* 230:16–17
12. Meyer, D., Larrieu, M. D. 1971. *Eur. J. Clin. Invest.* 1:425–31
13. Berglund, G. 1962. *Brit. J. Haematol.* 8:204–14
14. Piper, W., Schreier, M. H. 1964. *Thromb. Diath. Haemorrh.* 11:423–43
15. Bennett, E., Huehns, E. R. 1970. *Lancet* 2:956–58
16. Feinstein, D. I., Rapaport, S. I., Chong, M. In press
17. Zimmerman, T. S., Ratnoff, O. D., Powell, A. E. 1971. *J. Clin. Invest.* 50:244–54
18. Hoyer, L., Breckenridge, R. T. 1968. *Blood* 32:962–71
19. Feinstein, D., Chong, M., Kasper, C. K., Rapaport, S. I. 1969. *Science* 163: 1071–72
20. Denson, K. W. E., Biggs, R., Haddon, M. E., Borrett, R. Cobb, K. 1969. *Brit. J. Haematol.* 17:163–71
21. Meyer, D., Gray, L., Larrieu, M.-J. 1970. *Nouv. Rev. Fr. Hematol.* 10:619
22. Goudemand, M., Parquet-Gernez, A., Fecall, A. 1963. *Nouv. Rev. Fr. Hematol.* 10:627
23. Zimmerman, T. S., de la Pointe, L., Edgington, T. S. Unpublished
24. Nilsson, I. M., Blomback, M., Blomback, B. 1959. *Acta Med. Scand.* 164: 263–78
25. Cornu, P. M., Larrieu, M.-J., Caen, J., Bernard, J. 1963. *Brit. J. Haematol.* 9: 189–202
26. Bennett, B., Ratnoff, O. D., Levin, J. 1972. *J. Clin. Invest.* 51:2597–601
27. Holmberg, L., Nilsson, I. M. 1973. *N. Engl. J. Med.* 288:595–98
28. Legaz, M. E., Schmer, G., Counts, R. B., Davie, E. W. 1973. *J. Biol. Chem.* 248: 3946–55
29. Herschgold, E. J., Davison, A. M., Janzen, M. E. 1971. *J. Lab. Clin. Med.* 77:185–205

30. Marchesi, S. L., Shulman, N. R., Gralnick, H. R. 1972. *J. Clin. Invest.* 51: 2151–61
31. Van Mourik, J. A., Mochtar, I. A. 1970. *Biochim. Biophys. Acta.* 221:677–79
32. Stites, D. T., Hersgold, E. J., Portman, J. D., Fudenberg, H. H. 1971. *Science* 171: 196–97
33. Hoyer, L. W. 1972. *J. Lab. Clin. Med.* 80:822–33
34. Meyer, D., Lavergne, J. M., Larrieu, M.-J., Jasso, F. 1972. *Thromb. Res.* 1: 183–95
35. Bouma, D. N., Wigerinck, Y., Sixma, J. J., Van Mourik, J. A., Mouchtar, I. A. 1972. *Nature (New Biol.)* 236:104–6
36. Zimmerman, T. S., Ratnoff, O. D., Littell, A. S. 1971. *J. Clin. Invest.* 50: 255–58
37. Ratnoff, O. D., Bennett, B. 1973. *N. Engl. J. Med.* 288:342–45
38. Hoyer, L. Private communication
38a. Meyer, D. Private communication
39. Zimmerman, T. S., Edgington, T. S. 1973. *J. Exp. Med.* 138:1015
40. Weiss, H. J., Rogers, J., Brand, H. 1972. *Blood* 40:939
41. Weiss, H. J., Rogers, J., Brand, H. 1973. *Blood* 41:809–15
42. Meyer, D., Jenkins, C., Dreyfus, M., Larrieu, M.-J. 1973. *Nature* 243:293

Copyright 1974. All rights reserved

VASOPRESSIN IN OSMOTIC REGULATION IN MAN

❖7115

Gary L. Robertson, M.D.
Department of Medicine, Veterans Administration Hospital and University of Indiana Medical Center, Indianapolis, Indiana

INTRODUCTION

The important role of vasopressin in maintaining the osmotic constancy of body water was delineated over 20 years ago by the pioneering work of Verney, du Vigneaud, and others (1–3). Although the ensuing decades have seen significant advances in our understanding of certain aspects of vasopressin function (4), precise quantitative information on the behavior of this system in man has been rather limited. This has been due largely to the lack of simple and reliable methods for measuring vasopressin in blood at the low concentrations normally present under physiologic conditions. Recently, however, a number of sensitive and specific new radioimmunoassay methods for measuring vasopressin in plasma (5–7) and urine (8, 9) have been developed and are now beginning to yield a much clearer picture of this important homeostatic mechanism. This review focuses on some of the clinical information derived from these immunoassay studies and refers to earlier bioassay data only where comparisons seem particularly instructive.

NORMAL FUNCTION

Immunoassay-Bioassay Relationships

The plasma vasopressin values obtained by immunoassay methods appear to be in close, though not perfect, agreement with those reported from the bioassay laboratories (Table 1). The largest discrepancies seem to occur under hydropenic conditions, where significantly higher values have been reported by most of the bioassay laboratories. Although these differences could easily be due to unspecified clinical variables, such as posture, or to technical factors, such as nonuniformity of the reference standards or of the extent to which plasma vasopressin activity may have decayed during storage (7, 11), some consideration should also be given to the possibility that man may be capable of producing one or more antidiuretic hormones in addition to vasopressin (15, 16). Whatever the reason for the apparently higher bioassay

Table 1 Plasma vasopressin concentration in healthy adults in various states of water balance[a]

		Basal			Hydropenic			Hydremic		
Method	[Reference]	mOsm/kg	pg/ml	μU/ml	mOsm/kg	pg/ml	μU/ml	mOsm/kg	pg/ml	μU/ml
Immunoassay	[7]	287 ± 2	2.7 ± 1.4	(1.1 ± 0.6)	292 ± 2	5.4 ± 3.4	(2.2 ± 1.4)	282 ± 1	1.1 ± 0.8	(0.6 ± 0.3)
	[6]				288 ± 2	5.5 ± 0.3[b]	(2.2 ± 0.1)			
Bioassay	[14]						1.8 ± 0.7			
	[13]						4.6 ± 0.6			0.0
	[12]			[c]0.7 ± 1.0						
	[11]			2.7 ± 0.3			8.0 ± 1.0			0.0
	[10]			1.9 ± 0.3			6.5 ± 0.9			

[a] Samples were obtained during ad lib fluid intake (basal), after 8 to 10 hr of fluid deprivation (hydropenic), and 1 to 2 hr after rapid ingestion of water, 20 ml/kg body weight (hydremic). The subjects were recumbent in [7], [12], and [14]; seated in [11]; and in an unspecified posture for the other studies. Plasma vasopressin is expressed either in terms of weight (pg/ml) or biologic activity (μU/ml). Plasma osmolality is expressed in mOsm/kg. All values represent the mean ± the standard error of the mean.

[b] Corrected for extraction recovery.

[c] Corrected for whole blood to plasma.

values, however, there seems little doubt that the immunoassay methods now in use (6, 7) are quite specific for vasopressin and do not react significantly with oxytocin or any nonhormonal components of plasma.

Normal Control Mechanisms

In healthy recumbent adults, there is a direct and highly significant correlation between plasma vasopressin concentration and plasma osmolality (5–7). In one normal population (Figure 1), this relationship was described by the regression equation $y = 0.40\,[x–280]$, where y represents the plasma vasopressin concentration in pg/ml, x the plasma osmolality in mOsm/kg, 280 the threshold or intercept on the x or osmolality axis, and 0.4 the sensitivity or slope of the regression line. Two

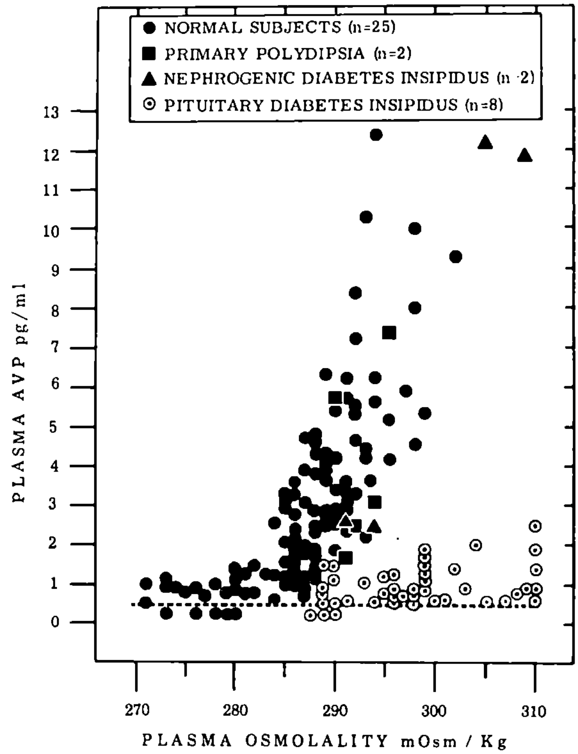


Figure 1 The relationship of plasma vasopressin to plasma osmolality in normal subjects and patients with polyuria of diverse etiologies. Samples were obtained from recumbent subjects in various states of water balance. The regression equation describing this relationship, $y = 0.4\ [x - 280]$ had a correlation coefficient of 0.52 ($P < .001$). The horizontal broken line indicates the sensitivity limit of the plasma vasopressin assay. Robertson et al (7).

interesting points emerge from this analysis. First, the sensitivity of the osmoregulatory system is such that a change in plasma osmolality as small as 1% (2.8 mOsm/kg) is sufficient to evoke a change in plasma vasopressin of about 1 pg/ml, an amount readily detectable by most assays and large enough to cause a pronounced change in urine osmolality (Figure 2). This extraordinary sensitivity, which fully confirms the original conclusions of Verney (1), means that plasma vasopressin values (or urine osmolalities) and their response to various drugs or clinical procedures can be interpreted properly only in conjunction with accurate measurements of plasma (17) osmolality.

An equally important characteristic of this osmoregulatory mechanism is its threshold value, i.e. the level of plasma osmolality below which there is little or no secretion of vasopressin. In the absence of hormonal stimulation, the capacity of normal kidneys to excrete free water is at least as great as the rate at which the ingestion of water can be sustained. For practical purposes, therefore, the threshold of the osmoreceptor mechanism determines the lower limit to which plasma osmolality can be depressed, no matter how large the water intake. Analysis of the data in Figure 1 indicates that in normal subjects this value averages out to be near 280

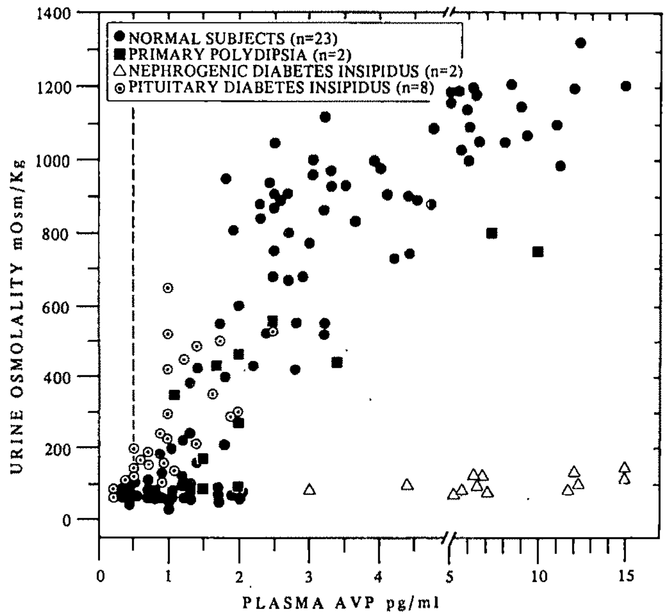


Figure 2 The relationship of plasma vasopressin to urine osmolality in normal subjects and patients with polyuria of diverse etiology. The regression equation describing this relationship, $y = 250\ [x - 0.25]$, had a correlation coefficient (r) of 0.77 ($P < .001$). The vertical broken line indicates the sensitivity limit of the plasma vasopressin assay. Robertson et al (7).

mOsm/kg, a result significantly lower than the value of 288 mOsm/kg previously determined for man by using free water clearance as an index of vasopressin activity (18). Although the reason for this discrepancy is not apparent, the latter value is probably much too high, since it exceeds mean basal plasma osmolality as reported by two other laboratories (Table 1). It seems likely, therefore, that the lower value of 280 more closely approximates the true osmotic threshold for vasopressin secretion in man.

A significant correlation can also be found between plasma vasopressin concentration and urinary osmolality (7, 12). In one group of subjects studied by immunoassay (Figure 2), this relationship appeared to be relatively linear at plasma vasopressin concentrations ranging from about 0.5 pg/ml, where urine was maximally dilute, to 5 pg/ml, where maximum concentration occurred. It is apparent from the steepness of the dose–response curve that a high degree of assay precision and sensitivity is necessary to study the relationship between vasopressin secretion and antidiuresis in man. The physiologically effective range of plasma vasopressin concentrations corresponds to a relatively narrow range of plasma osmolalities—from about 281 to 295 mOsm/kg—with the normal basal values (Table 1) situated somewhere near the middle of this zone. Thus, even a relatively short period of fluid restriction, which reduced body water by less than 2%, increased plasma vasopressin to the top of the dose response curve, while a standard water load, which expanded body water by less than 3%, depressed plasma vasopressin to levels permitting maximum diuresis (Table 1). The system is thus admirably suited for fine tuning of the tonicity of body fluids, since the full range of renal concentrating and diluting capacity can be brought rapidly into play in response to relatively small changes in water balance.

In normal subjects overhydration is not always associated with a complete absence of circulating vasopressin (Figure 1) even though the urine may be maximally dilute (Figure 2). Although the bioassay laboratories usually have reported plasma antidiuretic activity to be undetectable under these conditions (Table 1), the more sensitive immunoassays can often detect small but readily discernible amounts of activity that are physically indistinguishable from true vasopressin (7). Whether this residual activity is due to a failure to completely shut off secretion or to incomplete elimination of previously formed hormone remains to be determined.

It has long been known that certain nonosmotic factors, such as blood volume and blood pressure, may also influence vasopressin secretion (4), but the relative importance of these variables has never been precisely determined. This question is of particular importance in man since upright posture, which reduces intrathoracic blood volume by at least 10% (19), has been reported by different bioassay laboratories either to increase (20) or to have no effect (14) on plasma antidiuretic activity. Recent immunoassay studies have confirmed that upright posture does cause a rise in plasma vasopressin, but that this effect is significant only under hydropenic conditions (Table 2). Judging from this and other immunoassay studies (21), it would appear that a reduction in blood volume of at least 10% is necessary to effect vasopressin secretion in normally hydrated adults. Similarly, acute isovolemic re-

Table 2 The effect of posture on plasma vasopressin[a]

	Plasma Vasopressin, pg/ml		
	Upright	Recumbent	P
Basal	2.9 ± 0.2	2.6 ± 0.2	>0.2
Hydropenic	5.7 ± 0.7	4.8 ± 0.6	<0.005

[a] Values are the mean ± standard error (SE) plasma vasopressin concentration in 16 normal subjects before (basal) and after 12 to 16 hr of fluid deprivation (hydropenic). Mean (± SE) plasma osmolality was 286.8 ± 0.6 mOsm/kg and 290.4 ± 1.1 mOsm/kg in the basal and hydropenic states, respectively, and was not affected by posture. The probability value (*P*) was calculated using the t-test for paired samples (Robertson, G.L. and Athar, S., unpublished observations).

duction of blood pressure will also increase plasma vasopressin provided the change exceeds 5–10% of the resting recumbent value (7). Thus, both hypotension and hypovolemia can have important influences on vasopressin secretion but, because of the magnitude of the changes required, are probably less important than blood osmolality in the day to day regulation of vasopressin in man.

Undegraded vasopressin is also excreted in human urine at rates that appear to vary appropriately with the state of hydration (8, 9). Since the amounts of vasopressin involved are much greater than in plasma, this approach would seem to offer a way of assessing hormone function that is technically less demanding than the plasma assays. However, there is evidence that the urinary clearance of vasopressin in healthy adults varies considerably depending on the state of hydration and/or the solute excretion rate and, as a result, marked changes in vasopressin excretion can occur independently of changes in either the plasma concentration or secretion rate (22). As with several other polypeptide hormones, therefore, the rate of excretion of vasopressin may not always be a reliable index of its secretion and studies based on this variable should be interpreted with caution.

ABNORMAL FUNCTION

Diabetes Insipidus

Patients with vasopressin-responsive or pituitary diabetes insipidus can be readily distinguished from healthy subjects or patients with other kinds of polyuria by determination of plasma vasopressin (Figure 1). This distinction can be made most clearly when the patient is in a moderately hypertonic state (plasma osmolality >295 mOsm/kg) as after an appropriate period of fluid deprivation or the infusion of hypertonic saline. In response to these procedures, the plasma vasopressin concentration in these patients will usually rise, sometimes even to biologically active levels (Figure 2), but the magnitude of the vasopressin increase will almost always be distinctly subnormal relative to the level of plasma osmolality achieved (Figure 1). Thus, most patients with acquired pituitary diabetes appear to have some residual capacity to secrete vasopressin in response to osmotic stimulation, but in amounts too small to be physiologically useful. In contrast, patients with polyuria due either to nephrogenic diabetes insipidus or primary polydipsia are characterized by the presence of plasma vasopressin levels completely normal relative to plasma osmolality (Figure 1). Thus, neither the chronic underhydration nor overhydration characteristic of these conditions appears to alter the behavior of the osmoregulatory mechanism. As expected, patients with nephrogenic diabetes insipidus may be readily distinguished from those with primary polydipsia by the markedly abnormal relationship between plasma vasopressin and urinary osmolality that characterizes the former condition (Figure 2).

Immunoassay studies have yielded partially conflicting results regarding the mechanisms by which chlorpropamide improves antidiuresis in patients with pituitary diabetes insipidus (23, 24). While there seems to be general agreement that a major action of the drug is to potentiate the biologic effects of small amounts of endogenous vasopressin, one study based on measurements of urinary vasopressin has suggested that chlorpropamide may also stimulate hormone secretion (23), a conclusion not supported by measurements of vasopressin in plasma (24). The reason for the contradictory results in this situation has not been explained.

Inappropriate Antidiuresis

Both immunoassay (6, 25) and bioassay (26) studies have demonstrated that most patients with the clinical syndrome of inappropriate antidiuresis have abnormally high plasma vasopressin levels relative to the hypotonicity present. However, except for some cases due to ectopic production of the hormone by a tumor, the absolute concentration of plasma vasopressin in these patients tends to be in the same range as that found in healthy subjects (25,26), a finding that again emphasizes the importance of plasma osmolality measurements in all studies of vasopressin function. An unexpected and as yet unexplained observation in a few of these patients has been the presence of apparently normal osmoregulation of vasopressin despite persistent production of inappropriately concentrated urine (25). Further study of these interesting patients will be needed to determine if this phenomenon is due to an inability of the assay to detect subtle abnormalities in vasopressin secretion or whether the syndrome may occasionally result from other causes, such as abnormal production of one of the other antidiuretic substances recently postulated to be active in man (15, 16).

Literature Cited

1. Verney, E. B. 1947. *Proc. Roy. Soc. London B* 135:25–105
2. du Vigneaud, V. 1955. *Proc. 3rd Int. Congr. Biochem., Brussels,* 49–54
3. Berliner, R. W., Levinsky, N. G., Davidson, D. G., Eden, M. 1958. *Am. J. Med.* 24:730–44
4. Symposium on Antidiuretic Hormone. 1967. *Am. J. Med.* 42:651–827
5. Robertson, G. L., Klein, L. A., Roth, J., Gorden, P. 1970. *Proc. Nat. Acad. Sci. USA* 66:1298–1305
6. Beardwell, C. G. 1971. *J. Clin. Endocrinol. Metab.* 33:254–60
7. Robertson, G. L., Mahr, E. A., Athar, S., Sinha, T. 1973. *J. Clin. Invest.* 52:2340–52
8. Oyama, S.N., Kagan, A., Glick, S. M. 1971. *J. Clin. Endocrinol.* 33:739–44
9. Miller, M., Moses, A. M. 1972. *J. Clin. Endocrinol. Metab.* 34:537–45
10. Yoshida, S., Motohaski, K., Ibayashi, H., Okinaka, S. 1963. *J. Lab. Clin. Med.* 62: 279–85

11. Czaczkes, J. W., Kleeman, C. R., Koening, M. 1964. *J. Clin. Invest.* 43:1625–40
12. Moore, W. W. 1971. *Fed. Proc.* 30:1387–94
13. Ahmed, A. B. J., George, B. C., Gonzales-Auvert, C., Dingman, F. 1967. *J. Clin. Invest.* 46:111–23
14. Share, L. et al 1972. *J. Clin. Endocrinol. Metab.* 35:171–74
15. Pavel, S. 1970. *J. Clin. Endocrinol. Metab.* 36:369–71
16. Buckman, M.T., Peake, G. T. 1973. *Science* 181:755–57
17. Redetzki, H. M., Hughes, J. R., Redetzki, J. E. 1972. *Proc. Soc. Exp. Biol. Med.* 139:315–18
18. Moses, A. M., Streeten, D. H. P. 1967. *Am. J. Med.* 42:368–77
19. Gauer, O. H., Thron, H. L. 1965. *Handb. Physiol. Sect. II* 2409–39
20. Segar, W. E., Moore, W. W. 1968. *J. Clin. Invest.* 47:2143–51
21. Robertson, G. L., Mahr, E. A. 1972. *J. Clin. Invest.* 51:79a
22. Robertson, G. L. 1972. *Clin. Res.* 20:778
23. Moses, A. M., Numann, P., Miller, M. 1973. *Metab. Clin. Exp.* 22:59–66
24. Robertson, G. L., Mahr, E. A. 1971. *Proc. Fifty-third Annual Meeting Endocrine Society,* p. 125. Philadelphia: Lippincott. 284 pp.
25. Robertson, G. L., Mahr, E. A. 1972. *Proc. 4th Int. Congr. Endocrinol. Excerpta Med. Int. Congr. Ser. 256,* p. 47
26. Baumann, G., Lopez-Amor, E., Dingman, J. F. 1972. *Am. J. Med.* 52:19–24

Copyright 1974. All rights reserved

MEDICAL MANAGEMENT OF THE HYPERCALCEMIA OF MALIGNANCY[1]

◆7116

Leonard J. Deftos, M.D.
Departments of Medicine (Endocrinology), University of California and Veterans Administration Hospital, La Jolla, California

and

Robert Neer, M.D.
Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts

MECHANISMS OF THE HYPERCALCEMIA OF MALIGNANCY

Hypercalcemia is a common complication of cancer and has been reported in a wide variety of neoplastic processes (1). There are several mechanisms by which malignancy can produce hypercalcemia, the most common being the direct invasion of bone by tumor with subsequent destruction of bone mass and release of calcium into blood (2). In many cases, the involvement of bone by the malignancy is readily demonstrable by appropriate X-ray studies. However, invasion of bone by the cancer may be present even in the absence of X-ray changes and special procedures such as bone biopsy, marrow aspiration, or bone scanning may be necessary to demonstrate the presence of the malignancy in bone (3).

The hypercalcemia produced by the destruction of bone by malignancy can be aggravated in several ways. Patients with metastatic disease and, in fact, patients with many of the diseases described in this review, are confined to bed, and this immobilization itself may worsen the hypercalcemia. The mechanism of this effect is not firmly established and, in most instances, the hypercalcemia is probably only slightly aggravated. More devastating can be the effects of certain forms of treatment on the hypercalcemia complicating cancer metastatic to bone. There have been several reports of the rapid progression of hypercalcemia in patients with breast carcinoma metastatic to bone who are treated with estrogens, androgens, and progestins (4–6). The growth of these tumors was apparently stimulated by the exogenous administration of the hormones and resulted in greater destruction of bone. In some cases, the continuation of the hormonal treatment eventually led to regression of the tumor and the hypercalcemia (7). However, the continuation of hormone therapy under such circumstances is potentially dangerous to the patient.

---

[1] Supported by grants from the USPHS, the American Cancer Society, and the Veterans Administration.

Another mechanism by which cancer can cause hypercalcemia is through the production by the tumor of parathyroid hormone (PTH) or a parathyroid hormone-like substance (8). It has long been known that tumors can produce a substance that is similar or identical to parathyroid hormone. A wide variety of tumors have been shown to exhibit this potential, but carcinoma of the lung, especially squamous cell, and of the kidney have been especially common. In these cases, parathyroid hormone or a parathyroid hormone-like substance has been demonstrated in both tumor and blood by immunochemical means, primarily radioimmunoassay. Accordingly, these patients will differ from patients with hypercalcemia due to bony metastases since PTH levels in the latter group will be suppressed by the elevated blood calcium. However, PTH measurements cannot always reliably distinguish between these two groups of patients (1). Furthermore, Berson & Yalow pointed out that a significant percentage of patients with carcinoma of the lung may have elevated PTH levels in the absence of hypercalcemia (9).

It cannot be concluded that the PTH secreted by these tumors is structurally similar to normal PTH since an amount of hormone from such tumors sufficient for structural studies has not been isolated. Although genetic theory would not predict it, there is some immunochemical evidence to suggest that PTH produced by such tumors is structurally different from PTH produced by the parathyroid glands (10).

In addition to PTH, it has been suggested that some tumors may produce other biologically active substances that can cause hypercalcemia. Gordan reported the occurence of a vitamin D-like sterol in breast carcinomas (11). However, the significance of this observation and its relationship to the high incidence of hypercalcemia in breast carcinoma is unclear (12). The hypercalcemia that does occur in patients with breast carcinoma can often be explained on the basis of bone metastases by the tumor (13, 14).

Bioactive substances other than those related to PTH and vitamin D may also be secreted by certain tumors. Powell observed bone-resorbing activity not related to PTH in a number of tumors associated with hypercalcemia but without obvious metastases (15). A role for prostaglandins in the production of hypercalcemia via an increase in bone resorption has been suggested (16). In addition, there exist several experimental tumors in animals that are regularly associated with hypercalcemia without metastases but with increased bone resorption (17). Therefore, it is possible that as yet undiscovered or unappreciated substances produced by neoplasms may be responsible for some instances of malignancy-associated hypercalcemia. In this respect, the suggestion has been made that some malignant tumors may secrete a trophic hormone that stimulates the secretion of PTH (1). However, no convincing or direct evidence supports this intriguing hypothesis. The hypercalcemia of parathyroid carcinoma is, of course, due to the excess PTH production by this tumor, but this tumor has been reported to metastasize to bone (18).

An unusual mechanism by which malignant disease may cause hypercalcemia is the production of adrenal insufficiency by adrenal gland metastases (3). This form of hypercalcemia, also seen in other forms of adrenal insufficiency, probably does not represent a true increase in ionized calcium but results in increased binding of calcium by serum proteins. By a similar mechanism, the high levels of gamma globulin in myeloma may increase the total calcium without increasing ionized calcium (3). Myeloma can also produce actual hypercalcemia by direct invasion of bone (1). Finally, it should be kept in mind that many patients with malignancy have nausea and vomiting and are consequently dehydrated. This, too, can produce a spurious increase in total plasma calcium.

TREATMENT OF THE HYPERCALCEMIA OF MALIGNANCY

A wide variety of regimens is now available for the treatment of the hypercalcemia associated with malignancy. These agents work through several mechanisms and each method has its relative advantages and disadvantages as well as its indications and contraindications. An appreciation of these should result in a therapeutic choice that will be effective in the treatment of most forms of hypercalcemia.

Hydration

Dehydration is a common complication of hypercalcemia and malignancy because of frequently occuring nausea and vomiting. As a consequence, the contraction of the vascular space results in an even higher calcium concentration. However, it should be kept in mind that the rise in plasma calcium from dehydration is primarily attributable to an increase in the protein-bound calcium, probably not the ionized calcium (3). Nevertheless, since dehydration is so common and since rehydration can produce a marked clinical response, adequate hydration should be one of the first forms of treatment in hypercalcemia states. Saline is the agent of choice but the usual precautions should, of course, be taken regarding adequate renal and cardiopulmonary function. The latter considerations are especially important since large doses, up to 3 l of normal saline, may be necessary for effective therapy. In addition to being a better rehydrating agent than dextrose, sodium has another important action—it promotes the renal excretion of calcium. (19). The exact mechanism of this effect has not been firmly established but saline can promote the renal excretion of calcium to a degree sufficient to reduce the hypercalcemia. In many cases of mild hypercalcemia, rehydration with saline alone may be adequate to control the hypercalcemia (3). However, in most cases of significant hypercalcemia, additional forms of therapy will be necessary.

Calciuresis and Chelation

The effect of sodium on the renal excretion of calcium can be enhanced by the concomitant use of the diuretics furosemide and ethacrynic acid (3). Thiazide diuretics cannot be used since they may aggravate the hypercalcemia (20). These agents are given intravenously: ethacrynic acid in doses of up to 40 mg/1–2 hr, and furosemide in doses up to 100 mg/1–2 hr (21). The dose is modified by the therapeutic response. The results of such regimens can be quite dramatic, with a several milligram drop in calcium within several hours and a greater than one gram excretion of urinary calcium during the first 24 hours of treatment. This can occur despite the presence of some degree of renal failure. However, there must be very close patient observation during such treatment. The urinary losses of all electrolytes must be monitored very closely so that adequate intravenous replacement can be made for the other electrolytes excreted with the calcium, such as sodium, potassium, and magnesium. Although this regimen is feasible as an initial step, the degree of patient monitoring that is necessary to prevent overhydration and depletion of other ions often makes it prohibitive as a continuing form of therapy. Accordingly, the use of forced diuresis should be reserved for the initial treatment of significant hypercalcemia where the necessary professional and technical monitoring facilities are available for the frequent determination of plasma and urinary electrolytes, and should be followed by a more feasible and practical subsequent treatment program.

SULFATE, CITRATE, AND EDTA Sodium sulfate infusion is a potent calciuretic regimen that has found use in the treatment of hypercalcemia (22). It has a dual mechanism of action. Natriuresis occurs and promotes the urinary excretion of calcium as has been previously discussed. In addition, a complex formation occurs between calcium and sulfate in the urine and the excretion of this complex augments the calciuretic effect of the sodium. Consequently, the infusion of a liter of an isotonic solution (0.12 *M*) of sodium sulfate can lower blood calcium by approximately 1 mg%. Accordingly, relatively large volumes of sodium sulfate may be used to lower blood calcium in a severely hypercalcemic patient. Nausea can occur as a result of rapid infusion but even more limiting is the possibility of fluid overload which must be evaluated according to the patient's cardiopulmonary status. Sodium sulfate infusion can also cause the depletion of magnesium and potassium. However, this can be precluded by the addition to the solution of magnesium sulfate (0.002 *M*) and potassium sulfate (0.005 *M*) (3).

Although not extensively used, sodium sulfate probably represents a reasonable choice for the treatment of hypercalcemia in a patient with adequate renal and cardiopulmonary function (23). As with other calciuretic agents, the duration of the effect persists only during active therapy and the blood calcium will return toward pretreatment levels several hours after the infusion is stopped.

Citrate has also been used in a similar manner for the treatment of hypercalcemia. However, since citrate is metabolized its effects are more difficult to regulate and, therefore, it has not received wide application (24). Similarly, there are too many therapeutic alternatives to warrant the current use of another calcium chelator, EDTA, in the treatment of hypercalcemia (1, 3).

Inorganic Phosphate

Inorganic phosphate is one of the most effective and reliable treatments for hypercalcemia. It has long been known that raising the serum phosphate will decrease the serum calcium, and vice versa (25), but only recently has phosphate been reintroduced in treating hypercalcemia (23, 26).

Inorganic phosphate is commercially available in liquid, powder, and capsule form for oral use, and as a liquid for intravenous use. Preparations differ in their content of phosphorus and associated cations and pH (Table 1).

Oral phosphate is most useful for the chronic treatment of hypercalcemia. A dose of 2000 mg phosphorus daily can lower serum calcium by several milligrams. Several days are necessary for maximum effect and GI intolerance may become a problem. The latter can often be minimized by a gradually increased dose. Intravenous phosphate can be used for the acute control of hypercalcemia. A dose of 1500 mg phosphorous given over 6–8 hr will often lower blood calcium by several milligrams at the end of the infusion. In appropriate circumstances, retention enemas can be substituted for oral therapy.

Although other mechanisms may play a role, phosphate exerts its hypocalcemic effect primarily by the precipitation of calcium into bone (27). However, precipitation occurs in extraskeletal sites as well and this effect accounts for the most serious toxicity of phosphate therapy. Laboratory experiments clearly demonstrate that inorganic phosphate treatment of hypercalcemia produces calcium deposits in the heart, kidneys, and other tissues (28). In addition, metastatic calcification has been reported in patients receiving oral or intravenous phosphate therapy, especially the latter, for hypercalcemia (26). However, such observations are difficult to interpret since hypercalcemia itself can cause metastatic calcification. These reservations notwithstanding, in view of the severity of hypercalcemic symptoms in some patients, oral or intravenous phosphate to the point of hyperphosphatemia remains a justifiable emergency treatment.

Oral phosphate therapy is one of the few practical regimens available for the chronic treatment of hypercalcemia. It should be given in the lowest effective dose,

Table 1 Commercially available phosphate preparations for oral, rectal, and/or parenteral use

Product	1000 mg P_i[a]	meq Na^+	meq K^+
Fleet PhosphoSoda®, Liquid (C.B. Fleet Co)	6.7 ml	40	—
Neutra-Phos®, Powder (Willen Drug Co)	5 g (1 tsp)	28.5	28.5
Neutra-Phos®, Capsule (Willen Drug Co)	4 caps.	28.5	28.5
Neutra-Phos-K®, Capsule (Willen Drug Co)	4 caps.	—	57.0
Phos-Tabs®, Tablets (Davies, Rose-Hoyt)	6 tabs.	—	51.0
Hyper-Phos-K®, IV liquid (Davies, Rose-Hoyt)	15 ml	—	50
In-Phos®, IV liquid (Davies, Rose-Hoyt)	40 ml	65	8

[a] Inorganic phosphate doses are usually described in milligrams of *phosphorus* since clinical laboratories use such units to report serum and urine concentrations of inorganic phosphate.

and the serum inorganic phosphate should be kept under 5.5 mg% to minimize soft tissue calcification (27).

Mithramycin

Mithramycin is a cytotoxic substance derived from an actinomycete of the genus *Streptomyces* and successfully used in the treatment of certain testicular tumors. It has only limited effects on other malignancies, but striking and consistent effects on skeletal metabolism: it directly inhibits bone resorption (1). Accordingly, it lowers serum (and urine) calcium, even in normal subjects and patients with cancer but without apparent skeletal disease (29). Because of this effect, this drug has been used with increasing frequency in the treatment of hypercalcemia and has become an important therapeutic agent (30–32).

Mithramycin must be given intravenously, either as a freshly prepared injection or as a 4–24 hr infusion. The usual dose for the treatment of hypercalcemia is 25 μg/kg body weight, a dose lower than that used for treating tumors. Its hypocalcemic effect is manifest within 48 hr and can last up to several days. When the serum calcium begins to rise, repeated doses can be given. However, the serious side effects of mithramycin are more likely with repeated doses. The major side effects are thrombocytopenia (with normal bone marrow megakaryocytes), hepatocellular necrosis, and resultant hemorrhage. Azotemia and proteinuria can also occur. In some cases, hypocalcemia can become severe enough to be symptomatic. However, these toxic effects are rare when only one or two doses are used, and, with the exception of hemorrhage, can usually be reversed by stopping the drug (29–32).

Steroids

Glucocorticoids can be effective in the treatment of hypercalcemia. Although these agents do have a mild calciuretic effect, their primary mechanism of action is an antitumor effect. Accordingly, steroids are only effective in those neoplasms whose growth is inhibited by their administration. These most commonly include myeloma, lymphoma, and carcinoma of the breast (1, 3, 23).

Very large doses of steroids are often needed—up to several hundred milligrams of cortisone or its equivalent daily. It may take a week for the hypercalcemia to respond although in some cases a decrease in calcium may occur within days. The complications of steroid therapy are well known and preclude prolonged treatment with these drugs. Because of this and the variable effect of steroids, they are of limited usefulness in the treatment of hypercalcemia and should be reserved for those circumstances in which they exhibit an antitumor effect (33).

Calcitonin

Calcitonin, the hypocalcemic hypophosphatemic hormone, has found some application in the treatment of hypercalcemia (34). The peptide exerts its hypocalcemic effect by inhibiting bone resorption. Calcitonin also increases the renal clearance of calcium. Three species of the hormone, porcine, human, and salmon calcitonin, have been used for the treatment of hypercalcemia. Salmon calcitonin is the most potent calcitonin known and also has the longest duration of action (1, 35, 36).

The therapeutic effects of calcitonin in hypercalcemia have generally been moderate. It is effective by parenteral administration only, and the hypocalcemic effect is manifest within a few hours. Depending on the species used, the hormone must be given every 3–8 hr to be effective. Calcitonin has been less effective than phosphate or mithramycin in patients with hypercalcemia due to malignancy (37, 38). After several hours or days, escape from the hypocalcemic effects of calcitonin can occur (39). The effectiveness of calcitonin in experimental animals is related to the serum inorganic phosphate level. Phosphate loading enhances and phosphate depletion inhibits the hypocalcemic effect of the hormone. Accordingly, synergism of calcitonin and inorganic phosphate in patients with hypercalcemia warrants further study (1, 40).

Calcitonin is still an experimental drug and is not yet commercially available for the treatment of hypercalcemia. However, its remarkable freedom from toxic effects warrants its continued evaluation in the treatment of hypercalcemia.

Dialysis

A rapid and striking reduction in blood calcium concentration can be achieved by both peritoneal and hemodialysis, especially the latter. However, with the availability of so many other less complicated modes for the treatment of hypercalcemia, the indications for the use of dialysis are becoming less frequent (3).

CHOICE OF REGIMEN

It is difficult to make abstract recommendations about the treatment of the hypercalcemia of malignancy. Many modes of therapy are available and each has a different combination of mechanism of action and toxicity. Accordingly, the therapy must be tailored to the particular requirements of the individual patient. Even the decision to treat the hypercalcemia can be difficult. The management of severe hypercalcemia in a patient with terminal cancer raises many medical and philosophical issues that are well beyond the scope of this review. Decisions about therapy must also be influenced by the patient's symptoms. Some patients can become confused and symptomatic with only a slight elevation of blood calcium; others may tolerate large increases in blood calcium. Therefore, the following recommendations must be modified by the clinical situation.

Mild hypercalcemia ($<$12 mg%) can usually be managed by adequate hydration with sodium chloride. Moderate (12–15 mg%) or severe ($>$15 mg%) hypercalcemia often require more vigorous therapy. The selection of a therapy will be dependent upon the basic disease, the clinical situation, the serum inorganic phosphate level, and baseline renal, hepatic, and bone marrow function. If careful monitoring of fluid balance and central venous pressure is possible and cardiopulmonary decompensation not a threat, calciuresis should probably be attempted. If hyperphosphatemia is already present, oral or intravenous phosphate is likely to aggravate the tendency to metastatic calcification. Hypophosphatemia in a hypercalcemic patient is, on the other hand, a clear indication for supplemental phosphate therapy. Mithramycin will control hypercalcemia of various etiologies, independently of its antitumor properties. Its onset of action is somewhat slower than parenteral phosphate, but its reliability in lowering serum calcium levels is matched only by phosphate. It should be among the first agents considered for the therapy of severe hypercalcemia. Its usefulness is only limited by hepatic, renal, and bone marrow toxicity, which become more apparent with repeated doses.

Glucocorticoids should be reserved for those patients in whom an antitumor effect might be anticipated, i.e. those with carcinoma of the breast, multiple myeloma, leukemia, lymphoma. However, steroids lower the serum calcium concentration over several days and should not be the sole treatment for life-threatening hypercalcemia.

Although there have been reports of the successful treatment of hypercalcemia with calcitonin, the therapeutic role of this hormone has not been well established. Furthermore, it is not commercially available in this country and can only be used as an investigational drug. However, it has several characteristics that make it potentially useful in certain instances. Unlike hydration and diuresis, it can be used in the presence of impaired renal and cardiopulmonary function. Unlike mithramycin, it can be used in the presence of impaired hepatic and renal function. Unlike phosphate, it can be used in the presence of hyperphosphatemia. Therefore, calcitonin may be useful in special circumstances for the treatment of hypercalcemia and it should be available in medical centers which commonly deal with the problem of hypercalcemia and malignant disease. Dialysis can be recommended for the treatment of hypercalcemia only under exceptional circumstances.

The treatment of hypercalcemia of malignancy should be considered primarily as an adjunct to the definitive identification and treatment of the underlying disease process. If the underlying disease can be treated, the hypercalcemia will also respond to the treatment. If the underlying disease cannot be treated, it will be necessary to evaluate the possibility of the chronic treatment of the hypercalcemia. Most of the regimens discussed have side effects and/or impracticalities that limit their chronic application.

Literature Cited

1. Deftos, L. J., Potts, J. T. Jr. In *Duncan's Diseases of Metabolism*, ed. P. L. Bondy. Philadelphia: Saunders. 7th ed. In press
2. Muggia, F. O., Heinemann, H. O. 1970. *Ann. Intern. Med.* 73:281
3. Walser, M. 1970. *Mod. Treatment* 7:662
4. Becker, V. L. 1969. *Cancer* 24:610
5. Myers, W. P. L. et al 1956. *J. Am. Med. Assoc.* 101:127
6. Kaufman, R. J. et al 1964. *J. Clin. Endocrinol. Metab.* 24:1235
7. Kennedy, B. J. et al 1955. *Am. J. Med.* 19:337
8. Sherwood, L. M. et al 1967. *J. Clin. Endocrinol. Metab.* 26:140
9. Berson, S. A., Yalow, R. S. 1964. *Science* 154:907
10. Riggs, B. L. et al 1971. *J. Clin. Invest.* 50:2079–83
11. Gordan, G. S. et al 1966. *Science* 148:1226
12. Day, E. A., Malcolm, A. T., Beeler, M. F. 1969. *Metabolism* 18:646
13. Graham, W. P. et al 1973. *Surg. Gynecol. Obstet.* 117:709
14. Lafferty, F. W. 1966. *Medicine* 45:247

15. Powell, D. 1973. *N. Engl. J. Med.* 289:176
16. Levine, L. et al 1972. *Biochem. Biophys. Res. Commun.* 47:888–96
17. Tashjian, A. H. Jr. et al 1973. *Prostaglandins* 3:515
18. Sigurdsson, G., Woodhouse, N. J. Y., Taylor, S., Joplin, G. F. 1973. *Brit. Med. J.* 1:27
19. Walser, M. 1961. *Am. J. Physiol.* 200:1099
20. Duarte, C. G. et al 1971. *N. Engl. J. Med.* 284:828
21. Suki, W. N. et al 1970. *N. Engl. J. Med.* 283:836
22. Chakmakjian, J. H., Bethune, J. E. 1966. *N. Engl. J. Med.* 275:862
23. Fulmer, D. H. et al 1972. *Arch. Intern. Med.* 129:923
24. Kennedy, B. J. et al 1953. *Cancer Res.* 13:445
25. Bulger, H. A. et al 1930. *J. Clin. Invest.* 9:143
26. Goldsmith, R. S., Ingbar, S. H. 1966. *N. Engl. J. Med.* 274:1
27. Eisenberg, E. 1970. *N. Engl. J. Med.* 282:889
28. Schneeberger, E. E., Morrison, A. B. 1967. *Am. J. Pathol.* 50:549
29. Slayton, R. E. 1971. *Clin. Pharmacol. Ther.* 12:833
30. Singer, F. R. et al 1970. *N. Engl. J. Med.* 283:634
31. Perlia, C. P. et al 1970. *Cancer* 25:389
32. Elias, E. G., Evans, J. T. 1972. *Surgery* 71:631
33. Thalassinos, N. C., Joplin, G. F. 1970. *Lancet* 2:537
34. West, T. E. T. et al 1971. *Lancet* 1:675
35. Habener, J. F., Singer, F. R., Neer, R. M., Deftos, L. J., Potts, J. T. Jr. 1972. In *Calcium, Parathyroid Hormone and the Calcitonins,* ed. R. V. Talmage, P. L. Munson, 152–58. Amsterdam: Excerpta Medica
36. Habener, J. F., Singer, F. R., Deftos, L. J., Potts, J. T. Jr. 1972. *Endocrinology* 90:952–60
37. Kammerman, S., Canfield, R. 1970. *J. Clin. Endocrinol. Metab.* 31:70
38. Foster, G. V. et al 1966. *Lancet* 1:107
39. Neer, R. M. et al 1970. *Clin. Res.* 18:676
40. Editorial 1973. *Brit. Med. J.* 1:371

Copyright 1974. All rights reserved

BIOGENIC AMINES AND AFFECTIVE DISORDERS

❖7117

Joseph J. Schildkraut, M.D.
Neuropsychopharmacology Laboratory, Massachusetts Mental Health Center and
Department of Psychiatry, Harvard Medical School, Boston, Massachusetts

In recent years the study of biogenic amine metabolism in patients with affective disorders (depressions and manias) has been stimulated by the increasing evidence that drugs used in the treatment of these disorders produce changes in the metabolism of one of the monoamines (i.e. the catecholamines, norepinephrine, and dopamine, as well as the indoleamine serotonin) (1). These studies, which have demonstrated a number of different biochemical alterations in patients with affective disorders, have contributed substantially to our understanding of the underlying pathophysiology of these disorders. Aspects of this literature have been reviewed frequently during the past several years (1–16).

For a number of years investigators have recognized the possibility that different subtypes of depressive or manic disorders might exhibit different specific alterations in the metabolism of the monoamines. It was suggested that studies of biogenic amine metabolism might ultimately contribute to the development of a more meaningful biochemical classification of the affective disorders and a more rational approach to the treatment of these disorders (2, 17). Since I had the opportunity to prepare a fairly extensive review of this field for the *Annual Review of Pharmacology* last year (16), I do not repeat much of this material in the present review. Instead, I emphasize studies that have appeared during the past year, focusing principally on those areas of research that now appear most likely to yield biochemical criteria for classifying different types of affective disorders and predicting differential responses to various forms of treatment.

3-METHOXY-4-HYDROXYPHENYLGLYCOL

The alterations in norepinephrine metabolism produced by drugs used in the treatment of affective disorders have been studied extensively during the past decade (1). On the basis of the initial findings of these studies it was suggested that some, if not all, depressions may be associated with an absolute or relative deficiency of catecholamines, particularly norepinephrine, at functionally important receptor sites in the brain, whereas manias may be associated with an excess of such monoamines. This formulation has come to be known as the catecholamine hypothesis of affective disorders (2).

The major metabolite of norepinephrine in brain appears to be 3-methoxy-4-hydroxyphenylglycol (MHPG) or its sulfate conjugate (18–23), although all findings do not support this conclusion (24). MHPG, which is excreted in the urine, may derive in part from the peripheral sympathetic nervous system as well as the brain, and the fraction of urinary MHPG that derives from norepinephrine originating in the brain remains problematic (25, 26). As described below, considerable interest is currently focused on studies of the urinary excretion of MHPG in patients with affective disorders. However, some investigators have questioned whether MHPG (or any biogenic amine metabolite) measured in the urine can provide meaningful information about the activity of the central nervous system in patients with affective disorders (27).

In order to explore this issue, one recent study (28) examined the relationship of MHPG excretion to certain aspects of central nervous system activity as reflected by all-night electroencephalographic sleep recordings. In a small series of patients with affective disorders studied during drug-free periods, a statistically significant inverse correlation was observed between MHPG excretion and the amount of time spent in desynchronized, i.e. REM, sleep (D-time). When subgroups of affective disorders were examined separately, the inverse relationship between MHPG excretion and D-time was particularly evident in patients with manic-depressive (bipolar)[1] disorders. When taken in conjunction with pharmacological studies that have suggested an inverse relationship between D-time and central catecholaminergic activity (29–32), these findings appear to support the view that MHPG excretion may reflect central noradrenergic activity, particularly in patients with manic-depressive disorders (28).

A number of longitudinal studies of individual patients with naturally occurring or amphetamine-induced manic-depressive disorders (i.e. bipolar disorders) have found that the levels of urinary MHPG are relatively lower during depressions and higher during manic or hypomanic episodes than after clinical remissions (33–37). All findings, however, do not concur (38, 39). In one of these studies (39) the results may have been confounded by patients receiving psychoactive drugs (such as imipramine) which are known to cause pharmacologically induced alterations in MHPG excretion independent of effects on clinical state.

Although the urinary excretion of MHPG may vary with stress and changes in motor activity (40–42), it is unlikely that these factors account for the changes in MHPG excretion observed with changes of clinical state in manic-depressive patients, since in several of these studies the changes in MHPG excretion appeared

[1] The terms "manic-depressive" and "bipolar" are used synonymously to refer to patients with disorders characterized by at least one hypomanic or manic episode as well as one or more depressive episodes. These are to be differentiated from unipolar depressions in which there is no history of a prior hypomanic or manic episode.

to precede the changes in clinical state and the peaks in MHPG excretion did not appear to coincide with the peaks in motor activity (36, 37). Moreover, in another of these studies, reduced MHPG excretion was observed during agitated depressions in a small series of patients with manic-depressive disorders (33). All depressed patients, however, do not excrete comparably low levels of MHPG (43–46), and recent preliminary findings suggest that MHPG excretion may provide a biological criterion for classifying the depressive disorders and predicting the responses to specific forms of antidepressant pharmacotherapy.

In a recent study (46, 47) of a small group of patients with various clinically defined subtypes of depressive disorders examined prior to treatment with antidepressant drugs or electroconvulsive therapy, MHPG was significantly lower in patients with manic-depressive depressions (i.e. bipolar disorders with histories of hypomanias or manias) than in patients with chronic characterological depressions (i.e. dysphoric depressive syndromes with no history of hypomania or mania).[2] In a patient with a schizoaffective depression, MHPG excretion was lower than the mean for the manic-depressive depressions, while in a patient with a recurrent endogenous depression (but no history of hypomania or mania), MHPG excretion fell between the means for manic-depressive and chronic characterological depressions. There were no significant differences in the excretion of norepinephrine, normetanephrine, epinephrine, metanephrine, or 3-methoxy-4-hydroxymandelic acid (VMA), although the urinary excretion of norepinephrine tended to be lower in manic-depressive than in chronic characterological depressions. Thus, of the urinary catecholamines and metabolites measured in this study, only MHPG showed significant differences when values in the manic-depressive and chronic characterological depressions were compared (47). In this study, MHPG excretion did not appear to be related to the degree of retardation, agitation, or anxiety assessed on clinical rating scales (46, 47).

Another group of investigators (48) has recently reported that depressed patients classified as having a primary affective disorder (49, 50) excreted significantly less MHPG than did control subjects. In contrast, MHPG excretion in patients with undiagnosed types of affective disorders was not significantly different from control values. (A large variance in MHPG excretion was found in this undiagnosed group, suggesting that it may represent a heterogeneous sample.) Although MHPG excretion in depressed patients with bipolar depressive disorders tended to be lower than MHPG excretion in the other subgroups of patients with primary depressive disorders (i.e. unipolar recurrent and unipolar single episode), the differences among these subgroups were not statistically significant. MHPG excretion in the small subgroup of bipolar depressive disorders was significantly lower than the mean for control subjects. There were no statistically significant differences in normetanephrine, metanephrine, and VMA excretion when values in patients with primary affective disorders or any of the subtypes were compared with control values. In this study, MHPG excretion also did not appear to be related to differences in agitation or retardation (48).

---

[2] The clinical criteria for making these diagnoses are described in detail elsewhere (1).

Depressed patients who excreted relatively low levels of MHPG prior to treatment with imipramine or desmethylimipramine responded better to treatment with these antidepressants than did patients who excreted relatively higher levels of MHPG in one series of investigations (44). Other investigators did not confirm this finding, but some of the patients in this study were drug free for only five days before pretreatment MHPG levels were measured and residual drug effects may have influenced the initial MHPG values (51).

In a study of a small group of patients with depressive disorders, those patients who excreted relatively high levels of urinary MHPG prior to treatment responded better to treatment with amitriptyline than did patients with lower pretreatment levels of urinary MHPG (45, 52). These findings have recently been confirmed by other investigators (53). While clinical experience indicates that some depressed patients may respond to both amitriptyline and imipramine, whereas others may respond to neither, it has been suggested that the level of MHPG excreted in urine may provide a clinically useful biochemical criterion for choosing between amitriptyline and imipramine in the treatment of patients with depressive disorders (52).[3]

Free and conjugated MHPG have been demonstrated in human cerebrospinal fluid (CSF) (21, 54, 55), and several studies have recently examined the levels of MHPG in the lumbar CSF of patients with affective disorders. Since MHPG measured in lumbar CSF probably reflects norepinephrine metabolism in spinal cord as well as in brain (56), and since CSF levels of MHPG are determined by the rate of efflux of MHPG from the CSF as well as by the rate of production of MHPG in the central nervous system, the findings of these studies must be interpreted cautiously.

In a series of studies (54, 57), one group of investigators has found that CSF MHPG levels were significantly lower in depressed patients than in control subjects. Levels of CSF MHPG in these depressed patients were not related to the presence or absence of a history of mania. In a small group of patients studied during manic episodes, MHPG levels were not different from control values. Depressed patients who subsequently responded to tricyclic antidepressants were reported to have CSF MHPG values similar to nonresponders (the investigators did not indicate the specific tricyclic antidepressant drug or drugs used in this study). No correlations were observed between MHPG levels and clinical ratings of depression, agitation, retardation, psychosis, anxiety, or anger (57). However, differences in motor activity could conceivably contribute to differences in levels of CSF MHPG, since a statistically nonsignificant trend towards increases in CSF MHPG [as well as increases in CSF homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5HIAA)] was observed in one study after increased psychomotor activity was induced by asking patients to simulate mania or to be physically hyperactive (58).

Another group of investigators (39, 59, 60) reported that MHPG levels in CSF were not different from control values in a small heterogeneous group of depressed patients. Some manic patients showed markedly elevated levels of MHPG in an initial study by these investigators (59, 60), but in a subsequent report, based on a larger series of patients, these investigators indicated that the mean levels of CSF MHPG in manic patients were not significantly higher than control mean values (39). In these studies changes in clinical state in some bipolar manic-depressives were associated with marked changes in CSF MHPG levels corresponding to the direction of change in affective state (60, 39). As described above, similar changes in urinary MHPG have been observed in relation to changes in clinical state in a number of other longitudinal studies of manic-depressive patients.

---

[3] Recent neuropharmacological findings suggest a possible basis for such differences in the clinical effects of amitriptyline and imipramine (45).

In another small series of patients with recurrent (unipolar) depressions, manic-depressive depressions, and manias, there were no differences in the levels of MHPG between these various groups before treatment, nor did any significant changes occur after treatment. However, the investigators noted a wide scatter in the concentration of MHPG in the lumbar CSF of these patients (this study differs from others in that a spectrophotofluorometric rather than a gas chromatographic method was used to determine MHPG) (61).

These findings indicate that levels of MHPG in CSF as well as in urine may be decreased in some, but not all, depressed patients and increased in some patients with manias. Further investigation will clearly be required to confirm and extend these observations, as well as the findings suggesting that the urinary excretion of MHPG may provide a clinically useful criterion for classifying some types of depressive disorders and possibly for predicting differential responses to pharmacotherapy with one or another of the tricyclic antidepressant drugs.

HOMOVANILLIC ACID

Homovanillic acid (HVA), a deaminated O-methylated metabolite of dopamine, present in lumbar CSF, may provide information about the cerebral metabolism of dopamine (62), although some of the HVA in CSF may derive from capillaries in the brain (63). Interpretation of such data is further complicated because the concentration of HVA in lumbar CSF is considerably lower than in ventricular CSF, suggesting that there may be a transport system for the removal of HVA in the region of the fourth ventricle (62).

In a number of recent studies, depressed patients had lower baseline levels of HVA in the CSF[4] than did control subjects (58, 59, 64–70), but all studies do not concur (71, 72). A possible relationship between decreased baseline CSF HVA levels and decreases in motor activity in depressed patients has been suggested by some investigators (73), but such an association has not been observed in all studies (58, 59, 66). Two studies found no differences in baseline levels of HVA in CSF when levels in unipolar and bipolar depressions were compared (68, 71). In one study baseline levels of CSF HVA were significantly lower in unipolar depressions than in controls, whereas CSF HVA levels in bipolar depressions were within the normal range and significantly higher than in the unipolar group (74).

---

[4] The term "baseline level of HVA (or 5HIAA) in the CSF" is used to distinguish this measure from the level or accumulation of HVA (or 5HIAA) in the CSF after administration of probenecid (as described below).

In several studies, baseline CSF HVA levels in hypomanic and manic patients were found to be equal to or lower than control values (59, 64, 65, 68, 69, 74), but in one recent study increased baseline levels of HVA in CSF were observed in manic patients when compared with depressed patients or controls (71). Increased CSF HVA levels in patients with severe mania have been attributed to increased motor activity (58).

Because the rate of efflux of HVA or other metabolites from the CSF may vary over time and among subjects, measurements of the levels of these metabolites in lumbar CSF at an instant in time do not necessarily reflect the rates of production of these metabolites during a given time interval. However, such information about the rate of formation of acid monoamine metabolites may be obtained by blocking their efflux from the CSF with probenecid, a drug which inhibits transport of these acid metabolites (75). Accordingly, several recent studies have examined the accumulation of HVA in lumbar cerebrospinal fluid following the administration of probenecid in patients with affective disorders.

The accumulation of HVA in lumbar CSF following probenecid administration appears to be decreased in certain groups of depressed patients (as elaborated below) when compared with control values (64, 69, 71–73). However, levels of HVA following probenecid administration were higher in a diagnostically heterogeneous group of depressed patients than in a control population in one recent study (76). In two studies no differences were observed in HVA accumulation after probenecid administration when depressed and hypomanic patients were compared (69, 71).

One group of investigators has reported an association between the reduced probenecid-induced accumulation of HVA in the CSF of some depressed patients and a reduction in motor activity. In one study (73), the accumulation of HVA in patients with retarded depressions was significantly lower than control values, whereas the accumulation of HVA in patients with nonretarded depressions was slightly greater than control values. In another study (72), decreased levels of HVA in CSF after probenecid administration were observed in severely inhibited patients with neurotic as well as endogenous depressions. In this latter study, no differences were observed in CSF levels of HVA after probenecid administration when patients with unipolar and bipolar depressions were compared (72).

However, in a recent study by another group of investigators (69), depressed patients diagnosed as bipolar I[5] had significantly higher CSF levels of HVA following probenecid administration than did patients diagnosed as bipolar II or unipolar. In this study (69), depressed patients who showed an antidepressant response to

[5] The diagnosis of bipolar I is made by these investigators on the basis of a history of manic symptoms of sufficient severity to require hospitalization. The diagnosis of bipolar II is made on the basis of periods of abnormally increased activity or euphoria frequently at the termination of a depressive episode or during treatment with antidepressant drugs or electroconvulsive therapy (ECT).

treatment with lithium had significantly lower pretreatment levels of HVA in CSF after probenecid administration than did lithium nonresponders. However, some clarification will be needed since these investigators had previously reported that an antidepressant response to treatment with lithium is observed more frequently in patients with bipolar depressive disorders than in patients with unipolar depressions (77).

The findings of these studies suggest that the formation of HVA in the brain may be reduced in some patients with affective disorders. However, further investigation is required to explore the physiological basis of this phenomenon as well as to examine the relation between the levels or accumulation of HVA in lumbar CSF and aspects of clinical symptomatology or diagnostic subtypes.

5-HYDROXYINDOLEACETIC ACID

Measurement of 5-hydroxyindoleacetic acid (5HIAA), a deaminated metabolite of serotonin, in lumbar cerebrospinal fluid may yield information about the metabolism of serotonin in the brain (62), although some of the 5HIAA in lumbar CSF may come from the spinal cord (56, 78, 79). Accordingly, many investigators have studied 5HIAA in the CSF of patients with affective disorders.

Since the initial report that 5-hydroxyindole compounds were decreased in the cerebrospinal fluid of depressed patients (80), numerous studies have found statistically significant (68, 81–86) or nonsignificant decreases (65, 66) in baseline levels of CSF 5HIAA when depressed patients were compared with controls. However, in several recent studies depressed patients have been found to have essentially normal baseline levels of 5HIAA in the cerebrospinal fluid (59, 64, 67, 69, 71, 72). In most studies that observed a reduction in CSF 5HIAA levels in depressed patients, the reduction persisted after recovery (68, 74, 86), although a small rise to normal values upon recovery from depression was noted in one study of a small number of patients (82). It should be pointed out in relation to these findings in patients with depressive disorders that decreased baseline levels of 5HIAA have been observed in other psychiatric conditions including schizophrenic disorders (65, 81).

Baseline levels of 5HIAA in the CSF have been observed to be relatively higher in older subjects in two studies (87, 88), but in several other studies no such relation was observed between baseline CSF 5HIAA and age (66, 67, 89). In addition to age, a number of other factors might account for the variability observed in the many studies of CSF 5HIAA in depressions. These include: the nature of the control group with which depressed patients were compared; differences in the conditions under which the samples of cerebrospinal fluid were obtained; and differences in the techniques used for chemical determinations, including the possibility that some of these methods may be relatively nonspecific (90).

Possible differences in the diagnostic subgroups of the depressive disorders as well as in the clinical phenomenology of the patients examined in various studies may also be of importance in accounting for the differences in findings, and this has been explored by a number of investigators. In one study, depressed patients classified as psychotic (on the basis of the presence of delusions) had lower levels of 5HIAA in the CSF than did nonpsychotic depressed patients (68). In two recent studies, patients with unipolar (recurrent) depressions had lower baseline levels of 5HIAA in the CSF than did patients with bipolar manic-depressive depressions (68, 74), and in one of these studies normal baseline levels of 5HIAA in CSF were observed in patients with manic-depressive depressions (74). However, in another study there were no differences in CSF 5HIAA levels when patients with bipolar and unipolar depressions were compared (71). Other investigators have found no differences in CSF 5HIAA when patients with endogenous and neurotic depressions were compared (72). According to one recent investigation, the baseline levels of 5HIAA in the CSF appeared to show a bimodal distribution in patients with endogenous depressions, but a unimodal distribution in patients with nonendogenous depressions. In this study patients with relatively higher baseline levels of CSF 5HIAA tended to show better antidepressant responses to treatment with nortriptyline than did patients with lower baseline levels of CSF 5HIAA (88).

In some studies low baseline levels of CSF 5HIAA have been observed in hypomanic or manic patients both before treatment and after recovery (65, 82, 86), but pretreatment levels were not significantly lower in all of the studies. Other investigators, however, have observed normal or increased CSF 5HIAA levels in manic patients (64, 69, 71, 74, 81). Since 5HIAA levels in lumbar CSF have been found to increase after exercise or periods of simulated mania with increased psychomotor activity (58, 91) [possibly due to increased mixing of CSF from various levels during periods of physical activity (81)], the relatively low levels of CSF 5HIAA in manic or hypomanic patients observed by a number of investigators are of particular interest.

The accumulation of 5HIAA in the CSF following administration of probenecid was found to be lower in depressed patients than in control subjects in a number of recent studies (69, 71, 72, 83, 84, 92); the differences were statistically significant in most, but not all, of these studies. The possibility has been suggested that decreased accumulation of 5HIAA in the CSF after probenecid administration may be characteristic of only a subgroup of patients with endogenous depressions not necessarily distinguishable on the basis of psychopathological features or other differences in symptomatology (83, 84).

In one recent study (72), subnormal accumulation of 5HIAA in the CSF after probenecid administration (i.e. 5HIAA accumulation lower than the lowest value found in the control group) was observed more frequently in patients with endogenous depressions than in patients with neurotic depressions; moreover, within the group of endogenous depressions, subnormal accumulation of 5HIAA in the CSF occurred more frequently in bipolar than in unipolar depressions. In a preliminary study of a small number of depressed patients, these investigators have observed that patients with low pretreatment accumulation of 5HIAA in CSF after probenecid administration tended to show an antidepressant response to treatment with 5-hydroxytryptophan (5HTP), a precursor of serotonin, whereas patients with higher pretreatment accumulation of 5HIAA did not (92).

In another recent study no differences in the accumulation of 5HIAA in the CSF after probenecid administration were observed among depressed patients with bipolar I, bipolar II, or unipolar depressive disorders, but depressed patients who had favorable antidepressant responses to treatment with lithium carbonate had lower pretreatment accumulations of 5HIAA in the CSF following probenecid administration than did lithium nonresponders (69).

The levels of 5HIAA in CSF after probenecid administration in a group of patients with unipolar depressions were not different from control values in another study (76).

One group of investigators has reported that 5HIAA accumulation in the CSF after probenecid administration was significantly lower in manic patients than in control subjects (64, 71).

In one study no correlation was observed between baseline CSF 5HIAA levels and the increase in CSF 5HIAA after probenecid administration, but a positive correlation was observed between baseline levels of 5HIAA and HVA in the CSF as well as between the probenecid-induced increases in 5HIAA and HVA (72). Such correlations may simply reflect the common transport mechanisms involved in removing 5HIAA and HVA from the CSF. However, it is also possible that they may reflect some common factor in the metabolism of serotonin and dopamine in the central nervous system—for example, the common pathway of deamination by monoamine oxidase.

Because of a number of unresolved issues, the interpretation of data on the accumulation of acid monoamine metabolites in lumbar CSF following probenecid administration is problematic (93–95). These issues, which are also discussed elsewhere, include possible variations in the effects of probenecid from subject to subject (including differences in CSF probenecid levels or differences in the degree of blockade produced by a given concentration of probenecid); possible individual variations in transit time for 5HIAA (or HVA) to pass from brain to lumbar CSF; possible interindividual variations in the rate of transport of acid monoamine metabolites out of CSF; variations in the extent to which the transport of these metabolites is inhibited by the maximum dose of probenecid which may be administered to human subjects; possible variations in the volume of the cerebrospinal fluid; and possible effects of probenecid on monoamine metabolism apart from the inhibition of transport of acid metabolites (96, 97). Moreover, since 5HIAA may not be the sole metabolite of cerebral serotonin (just as HVA is not the sole metabolite of dopamine), the possible contribution of the corresponding alcohol derivatives or other metabolites must also be considered when interpreting these findings.

Despite some unresolved problems that prevent definitive interpretation, studies of the baseline levels of 5HIAA and HVA as well as the probenecid-induced accumulations of these acid monoamine metabolites in lumbar CSF of patients with affective disorders have generated a number of interesting clues concerning the underlying biochemical substrates of the affective disorders, and further investigation in this area is clearly indicated. Of particular importance are studies to ascertain whether such measures of acid monoamine metabolites in the CSF may have practical clinical applications in classifying patients with depressive disorders and predicting differential responses to treatment.

DOPAMINE-β-HYDROXYLASE ACTIVITY

Dopamine-β-hydroxylase activity was determined in serum or plasma of patients with affective disorders in two recent studies (98, 99). No differences were observed when values in manic or depressed patients were compared to control values, and there were no apparent differences in dopamine-β-hydroxylase activity in the subtypes of depressive disorders examined in these two studies. While these studies provide no evidence to suggest that serum or plasma dopamine-β-hydroxylase activity may be of value in differentiating among types of depressive disorders, further studies using other assay procedures may be indicated, since recent reports have suggested that radioimmunoassays might provide more physiologically relevant information than the enzymatic assays used heretofore (100, 101).

CATECHOL-O-METHYL TRANSFERASE ACTIVITY

In a series of studies, catechol-O-methyl transferase activity was determined in red blood cells of patients with primary affective disorders (102, 103). Red blood cell catechol-O-methyl transferase activity was significantly lower in women with unipolar depressions than in controls, whereas intermediate values were observed in women with bipolar illnesses. (Red blood cell catechol-O-methyl transferase activity in women with schizophrenia was not different from control values.) Within the group of women with primary affective disorders, red blood cell catechol-O-methyl transferase activity was independent of the phase of the illness (depression or mania) and did not change with recovery. In contrast to the differences observed in women with primary affective disorders, no differences in red blood cell catechol-O-methyl transferase activity were found among comparable diagnostic groups of male patients. Further studies are required to confirm these findings which suggest that there may be differences in red blood cell catechol-O-methyl transferase activity in women with different types of depressive disorders.

MONOAMINE OXIDASE ACTIVITY

The conversion of orally administered radioactive serotonin to radioactive 5HIAA recovered in urine was examined in a group of depressed patients (clinical subtypes unspecified) and normal control subjects in a study performed a number of years ago. There were no differences between the groups, and the investigators concluded that monoamine oxidase functioned normally in depressed patients (104). However, more recent studies suggest that there may be specific alterations of monoamine oxidase activity in certain types of depressive disorders.

One group of investigators recently reported that platelet monoamine oxidase activity was significantly higher in a large heterogeneous group of depressed patients than in a group of normal subjects matched for age (105). They also observed that there was a progressive increase in monoamine oxidase activity in human hindbrain, plasma, and platelets with advancing age and that women had higher mean platelet monoamine oxidase activity than men at all age levels and higher mean plasma monoamine oxidase activity after age 40. The mean hindbrain monoamine oxidase activity in women was slightly but not significantly greater than in men. Levels of norepinephrine in the hindbrain (obtained at necropsy from patients who had died from a variety of causes) decreased significantly with advancing age and the levels of norepinephrine in hindbrain correlated negatively with hindbrain monoamine oxidase activity. Neither serotonin nor 5HIAA levels in hindbrain correlated significantly with age, but levels of 5HIAA were positively correlated with hindbrain monoamine oxidase activity (106, 107). These findings may provide clues to explain the generally greater frequency of certain types of depressive illnesses in women than in men and the increasing incidence of some types of depressive illnesses with advancing age in both sexes.

In another study, plasma monoamine oxidase activity was significantly higher in a group of premenopausal depressed women than in a group of control subjects. The clinical subtypes of depressive disorders under investigation in this study were not clearly specified but the investigators noted that the study did not include "schizophrenic, psychotic, manic, reactive, or involutional depressives" (108).

Platelet monoamine oxidase activity was found in a recent study to be significantly lower in bipolar depressed patients than in unipolar depressed patients or normal controls of similar age and sex distribution (109). Patients with unipolar depressions had slightly higher platelet monoamine oxidase activity than controls, but this difference was not statistically significant. Platelet monoamine oxidase activity did not appear to vary with the phase of the illness in patients with manic-depressive disorders. There was a high negative correlation between platelet monoamine oxidase activity and tryptamine excretion, with bipolar patients excreting significantly more tryptamine than patients with unipolar depressions. Preliminary results from a further study indicate that the false transmitter octopamine (which accumulates in platelets after treatment with monoamine oxidase inhibitors) is present in platelets of patients with endogenously reduced monoamine oxidase activity, particularly patients with bipolar depressions, and it has been suggested that endogenous false transmitters might play a role in the pathophysiology of some types of depressive disorders (110).

Other investigators have reported that after infusion with radioactive norepinephrine, patients with retarded depressions classified as manic-depressives were found to have an elevated ratio of radioactive amines to deaminated metabolites in the urine when compared to normal controls or patients with agitated unipolar depressions (111, 112). While many factors, including an alteration in the disposition of the infused radioactive norepinephrine, could account for these results, these findings would be compatible with a decrease in the deamination of norepinephrine (or normetanephrine) in the manic-depressive patients.

Further studies have indicated that schizophrenic patients also have reduced platelet monoamine oxidase activity when compared with controls (113). Reduced platelet monoamine oxidase activity may reflect a genetically determined vulnerability to schizophrenia rather than the presence of the illness itself, since, in monozygotic twin pairs discordant for schizophrenia, the monoamine oxidase activities of both the schizophrenic and nonschizophrenic co-twins were significantly lower than in normal subjects and monoamine oxidase activity was highly correlated between twins (114). Similarly monozygotic twin pairs free of physical or psychiatric disorders showed significantly smaller differences in platelet monoamine oxidase activities than did dizygotic twin pairs or age and sex matched control pairs (115). The findings of reduced platelet monoamine oxidase activity in schizophrenias as well as in manic-depressive disorders suggest that there may be biochemical similarities between these groups of disorders. The difficulties in making clear-cut clinical distinctions between certain types of manic-depressive and schizophrenic disorders are generally recognized among psychiatrists and have been documented in several recent papers (116, 117).

Findings from one recent study indicate that at least some depressed patients may have abnormal serum monoamine oxidase isoenzyme patterns. These preliminary data also suggest that serum monoamine oxidase isoenzyme patterns may differ according to the subtypes of depressive disorders (118).

It is difficult to compare the findings of these various studies since different substrates were used in the assays of monoamine oxidase activity. Moreover, further investigation is required to determine whether platelet (or plasma) monoamine oxidase activity provides an index of the monoamine oxidase activities in other tissues, particularly the brain, which may have different isoenzymes. However, in the aggregate, the findings of these studies raise the possibility that the determination of monoamine oxidase activities or isoenzyme patterns may be of value in differentiating various subtypes of depressive disorders. Moreover, taken in conjunction with studies showing that genetic factors may influence platelet monoamine oxidase activity, these findings offer promising leads for further studies of the biochemical genetics of affective disorders.

CONCLUSION

The clinical as well as biological heterogeneity of the affective disorders has long been recognized in psychiatry, and this heterogeneity has been evidenced in recent years by the differential responses of various types of depressions to one or another form of treatment (1). The findings of a number of recent studies of biogenic amine metabolism in patients with affective disorders, summarized in this review, have suggested important clues to the different pathophysiological substrates underlying this heterogeneity, and these findings appear to provide some support for the possibility, put forth a number of years ago, that different subgroups of patients with depressive disorders may exhibit different specific abnormalities in the metabolism of norepinephrine or other biogenic amines (2). Moreover, these findings appear compatible with the suggestion that, in patients with affective disorders, certain changes in biogenic amine metabolism may occur in association with changes in affective state, whereas other abnormalities in monoamine metabolism may represent enduring constitutional factors (119).

Many recent studies have attempted to examine biochemical variables in relation to existing clinical categories such as endogenous, nonendogenous, chronic characteriological, manic-depressive (bipolar), and unipolar depressions. However, these clinical categories are not necessarily defined in identical terms by all investigators and it is, therefore, difficult to compare findings from various studies. Furthermore, since it is generally agreed that these clinically defined categories do not necessarily represent biologically homogeneous entities, it may prove to be more strategic to examine the possibility that various biochemical criteria, possibly in combination with certain specific clinical criteria, may serve as independent variables for classifying various types of depressive disorders. Such an approach could conceivably lead to new categories that are biologically more meaningful and therapeutically more relevant than those defined under any currently available system for the clinical classification of depressive disorders. Many investigators now envisage the development of a psychiatric nosology based not only on the clinical phenomenology of the affective disorders but also upon a knowledge of the biochemical mechanisms underlying these phenomena. Moreover, one can foresee the possibility of biochemical tests soon becoming as routine in the diagnostic workup of patients with affective disorders as they are now in the evaluation of patients with endocrinopathies or other types of metabolic disorders.

ACKNOWLEDGMENTS

I wish to thank Mrs. Gladys Rege and Miss Josna Rege for their assistance in the preparation of this review. This work was supported in part by USPHS Grant No. MH 15,413 awarded by the National Institute of Mental Health.

Literature Cited

1. Schildkraut, J. J. 1970. *Neuropsychopharmacology and the Affective Disorders*. Boston: Little, Brown
2. Schildkraut, J. J. 1965. *Am. J. Psychiat.* 122:509–22
3. Schildkraut, J. J., Kety, S. S. 1967. *Science* 156:21–30
4. Durell, J., Schildkraut, J. J. 1966. In *American Handbook of Psychiatry*, ed. S. Arieti, 3:423–57. New York: Basic Books
5. Bunney, W. E. Jr., Davis, J. M. 1965. *Arch. Gen. Psychiat.* 13:483–94
6. Prange, A. J. Jr. 1964. *Dis. Nerv. Syst.* 25:217–21
7. Coppen, A. 1967. *Brit. J. Psychiat.* 113:1237–64
8. Weil-Malherbe, H. 1967. *Advan. Enzymol.* 29:479–553
9. Bueno, J. R., Himwich, H. E. 1967. *Psychosomatics* 8:82–94
10. Himwich, H. E. 1970. In *Biochemistry, Schizophrenia and Affective Illnesses*, ed. H. E. Himwich, 230–82. Baltimore: Williams & Wilkins
11. Glassman, A. 1969. *Psychosom. Med.* 31:107–14
12. Lapin, I. P., Oxenkrug, G. F. 1969. *Lancet* 1:132–36
13. Praag, H. M. van. 1969. *Pharmakopsychiat. Neuro Psychopharmakol.* 2: 151–60
14. Davies, B. 1969. *Med. J. Aust.* 1:557–65
15. Davis, J. M. 1970. *Int. Rev. Neurobiol.* 12:145–75
16. Schildkraut, J. J. 1973. *Ann. Rev. Pharmacol.* 13:427–54
17. Schildkraut, J. J., Schanberg, S. M., Kopin, I. J., Durell, J. 1969. In *Aspects of Depression*, ed. E. Shneidman, M. J. Ortega, 73–141. Boston: Little, Brown
18. Axelrod, J., Kopin, I. J., Mann, J. D. 1959. *Biochim. Biophys. Acta* 36:576
19. Mannarino, E., Kirshner, N., Nashold, B. S. Jr. 1963. *J. Neurochem.* 10:373–79
20. Schanberg, S. M., Schildkraut, J. J., Breese, G. R., Kopin, I. J. 1968. *Biochem. Pharmacol.* 17:247–54
21. Schanberg, S. M., Breese, G. R., Schildkraut, J. J., Gordon, E. K., Kopin, I. J. 1968. *Biochem. Pharmacol.* 17:2006–8
22. Maas, J. W., Landis, D. H. 1968. *J. Pharmacol. Exp. Ther.* 163:147–62

23. Wilk, S., Watson, E. In *Frontiers in Catecholamine Research—Third International Catecholamine Symposium*, ed. E. Usdin, S. Snyder. New York: Pergamon. In press
24. Chase, T. N., Breese, G. R., Gordon, E. K., Kopin, I. J. 1971. *J. Neurochem.* 18:135–40
25. Maas, J. W., Dekirmenjian, H., Garver, D., Redmond, D. E. Jr., Landis, H. D. 1972. *Brain Res.* 41:507–11
26. Breese, G. R. et al 1972. *Nature New Biol.* 240:286–87
27. Angrist, B., Shopsin, B., Gershon, S. 1972. *Psychopharmacologia* 26:1–9
28. Schildkraut, J. J., Keeler, B. A., Papousek, M., Hartmann, E. 1973. *Science* 181:762–64
29. Hartmann, E., Bridwell, T. J., Schildkraut, J. J. 1971. *Psychopharmacologia* 21:157–64
30. Hartmann, E., Chung, R., Draskoczy, P. R., Schildkraut, J. J. 1971. *Nature* 233: 425–27
31. Wyatt, R. J. 1972. *Biol. Psychiat.* 5:33–64
32. Kupfer, D. J., Bowers, M. B. Jr. 1972. *Psychopharmacologia* 27:183–90
33. Greenspan, K. et al 1970. *J. Psychiat. Res.* 7:171–83
34. Schildkraut, J. J., Watson, R., Draskoczy, P. R., Hartmann, E. 1971. *Lancet* 2: 485–86
35. Schildkraut, J. J., Keeler, B. A., Rogers, M.P., Draskoczy, P.R. 1972. *Psychosom. Med.* 34:470; erratum 1973. *Psychosom. Med.* 35:274
36. Bond, P. A., Jenner, F. A., Sampson, G. A. 1972. *Psychol. Med.* 2:81–85
37. Jones, F. D., Maas, J. W., Dekirmenjian, H., Fawcett, J. A. 1973. *Science* 179:300–2
38. Bunney, W. E. Jr., Goodwin, F. K., Murphy, D. L., House, K. M., Gordon, E. K. 1972. *Arch. Gen. Psychiat.* 27:304–9
39. Shopsin, B., Wilk, S., Gershon, S., Roffman, M., Goldstein, M. See Ref. 23
40. Rubin, R. T., Miller, R. G., Clark, B. R., Poland, R. E., Arthur, R. J. 1970. *Psychosom. Med.* 32:589–97
41. Maas, J. W., Dekirmenjian, H., Fawcett, J. 1971. *Nature* 230:330–31
42. Ebert, M. H., Post, R. M., Goodwin, F. K. 1972. *Lancet* 2:766
43. Mass, J. W., Fawcett, J. A., Dekirmenjian, H. 1968. *Arch. Gen. Psychiat.* 19:129–34
44. Maas, J. W., Fawcett, J. A., Dekirmenjian, H. 1972. *Arch. Gen. Psychiat.* 26: 252–62
45. Schildkraut, J. J., Draskoczy, P. R., Gershon, E. S., Reich, P., Grab, E. L. 1971. In *Brain Chemistry and Mental Disease*, ed. B. T. Ho, W. M. McIsaac, 215–36. New York: Plenum
46. Schildkraut, J. J., Keeler, B. A., Grab, E. L., Kantrowich, J., Hartmann, E. 1973. Lancet 1:1251–52
47. Schildkraut, J. J. See Ref. 23
48. Mass, J.W., Dekirmenjian, H., Jones, F. See Ref. 23
49. Robins, E., Guze, S. B. 1972. In *Recent Advances in the Psychobiology of the Depressive Illnesses*, ed. T. A. Williams, M. M. Katz, J. A. Shield Jr., 283–93. Washington: GPO
50. Feighner, J. P. et al 1972. *Arch. Gen. Psychiat.* 26:57–63
51. Prange, A. J. Jr. 1972. *J. Psychiat. Res.* 9:187–206
52. Schildkraut, J. J. 1973. *Am. J. Psychiat.* 130:695–99
53. Beckmann, H., Goodwin, F. K. Personal communication
54. Gordon, E. K., Oliver, J. 1971. *Clin. Chim. Acta* 35:145–50
55. Wilk, S., Davis, K. L., Thacker, S. B. 1971. *Anal. Biochem.* 39:498–504
56. Post, R. M., Goodwin, F. K., Gordon, E., Watkin, D. M. 1973. *Science* 179:897–99
57. Post, R. M., Gordon, E. K., Goodwin, F. K., Bunney, W. E. Jr. 1973. *Science* 179:1002–3
58. Post, R. M., Kotin, J., Goodwin, F. K., Gordon, E. K. 1973. *Am. J. Psychiat.* 130:67–72
59. Wilk, S., Shopsin, B., Gershon, S., Suhl, M. 1972. *Nature* 235:440–41
60. Shopsin, B., Wilk, S., Gershon, S., Davis, K., Suhl, M. 1973. *Arch. Gen. Psychiat.* 28:230–33
61. Ashcroft, G. W. et al 1971. *Changes in the glycol metabolites of noradrenaline in affective illness.* Presented at Fifth World Congress of Psychiatry, Mexico City
62. Moir, A. T. B., Ashcroft, G. W., Crawford, T. B. B., Eccleston, D., Guldberg, H. C. 1970. *Brain* 93:357–68
63. Bartholini, G., Tissot, R., Pletscher, A. 1971. *Brain Res.* 27:163–68
64. Roos, B.E., Sjöström, R. 1969. *Pharmacol. Clin.* 1:153–55
65. Bowers, M. B. Jr., Heninger, G. R., Gerbode, F. 1969. *Int. J. Neuropharmacol.* 8:255–62
66. Papeschi, R., McClure, D. J. 1971. *Arch. Gen. Psychiat.* 25:354–58

67. Nordin, G., Ottosson, J. O., Roos, B. E. 1971. *Psychopharmacologia* 20:315–20
68. Mendels, J., Frazer, A., Fitzgerald, R. G., Ramsey, T. A., Stokes, J. W. 1972. *Science* 175:1380–82
69. Goodwin, F. K., Post, R. M., Dunner, D. L., Gordon, E. K. 1973. *Am. J. Psychiat.* 130:73–79
70. Brodie, H. K. H., Sack, R., Siever, L. In *Serotonin and Behavior,* ed. J. Barchas, E. Usdin. New York: Academic. In press
71. Sjöström, R., Roos, B.E. 1972. *Eur. J. Clin. Pharmacol.* 4:170–76
72. Praag, H. M. van, Korf, J., Schut, D. 1973. *Arch. Gen. Psychiat.* 28: 827–31
73. Praag, H. M. van, Korf, J. 1971. *Psychopharmacologia* 19:199–203
74. Medical Research Council, Brain Metabolism Unit. 1972. *Lancet* 2:573–77
75. Neff, N. H., Tozer, T. N., Brodie, B. B. 1967. *J. Pharmacol. Exp. Ther.* 158:214–18
76. Bowers, M. B. Jr. 1972. *Psychopharmacologia* 23:26–33
77. Goodwin, F. K., Murphy, D. L., Dunner, D. L., Bunney, W. E. Jr. 1972. *Am. J. Psychiat.* 129:44–47
78. Bulat, M., Zivkovic, B. 1971. *Science* 173:738–40
79. Weir, R. L., Chase, T. N., Ng, L. K. Y., Kopin, I. J. 1973. *Brain Res.* 52:409–12
80. Ashcroft, G. W., Sharman, D. F. 1960. *Nature* 186:1050–51
81. Ashcroft, G. W. et al 1966. *Lancet* 2:1049–52
82. Dencker, S. J., Malm, U., Roos, B.E., Werdinius, B. 1966. *J. Neurochem.* 13:1545–48
83. Praag, H. M. van, Korf, J., Puite, J. 1970. *Nature* 225:1259–60
84. Praag, H. M. van, Korf, J. 1971. *Psychopharmacologia* 19:148–52
85. McLeod, W. R., McLeod, M. S. 1971. *Aust. NZ J. Psychiat.* 5:289–95
86. Coppen, A., Prange, A. J. Jr., Whybrow, P. C., Noguera, R. 1972. *Arch. Gen. Psychiat.* 26:474–78
87. Bowers, M. B., Gerbode, F. A. 1968. *Nature* 219:1256–57
88. Asberg, M., Bertilsson, L., Tuck, D., Cronholm, B., Sjoqvist, F. 1973. *Clin. Pharm. Therap.* 14:277–87
89. Goodwin, F. K., Post, R. M. See Ref. 70
90. Wilk, S., Green, J. P. 1972. *J. Neurochem.* 19:2893–95
91. Fotherby, K., Ashcroft, G. W., Affleck, J. W., Forrest, A. D. 1963. *J. Neurol. Neurosurg. Psychiat.* 26:71–73
92. Praag, H. M. van, Korf, J., Dols, L. C. W., Schut, T. 1972. *Psychopharmacologia* 25:14–21
93. Bowers, M. B. Jr. 1972. *Neuropharmacology* 11:101–11
94. Sjöström, R. 1972. *Psychopharmacologia* 25:96–100
95. Praag, H. M. van, Korf, J. See Ref. 70
96. Tagliamonte, A., Tagliamonte, P., Perez-Cruet, J., Stern, S., Gessa, G. L. 1971. *J. Pharmacol. Exp. Ther.* 177:475–80
97. Korf, J., Praag, H. M. van, Sebens, J. B. 1972. *Brain Res.* 42:239–42
98. Shopsin, B., Freedman, L. S., Goldstein, M., Gershon, S. 1972. *Psychopharmacologia* 27:11–16
99. Wetterberg, L., Aberg, H., Ross, S. B., Froden, O. 1972. *Scand. J. Clin. Lab. Invest.* 30:283–89
100. Rush, R. A., Nagatsu, T., Geffen, L. B., Udenfriend, S. 1973. *Fed. Proc.* 32: 707
101. Geffen, L. B., Rush, R. A. See Ref. 23
102. Cohn, C. K., Dunner, D. L., Axelrod, J. 1970. *Science* 170:1323–24
103. Dunner, D. L., Cohn, C. K., Gershon, E. S., Goodwin, F. K. 1971. *Arch. Gen. Psychiat.* 25:348–53
104. Feldstein, A., Hoagland, H., Wong, K. K., Oktem, M. R., Freeman, H. 1964. *Am. J. Psychiat.* 120:1192–94
105. Nies, A., Robinson, D. S., Ravaris, C. L., Davis, J. M. 1971. *Psychosom. Med.* 33:470
106. Robinson, D. S., Davis, J. M., Nies, A., Ravaris, C. L., Sylwester, D. 1971. *Arch. Gen. Psychiat.* 24:536–39
107. Robinson, D. S. et al 1972. *Lancet* 1:290–91
108. Klaiber, E. L., Broverman, D. M., Vogel, W., Kobayashi, Y., Moriarty, D. 1972. *Am. J. Psychiat.* 128: 1492–98
109. Murphy, D. L., Weiss, R. 1972. *Am. J. Psychiat.* 128:1351–57
110. Murphy, D. L. 1972. *Am. J. Psychiat.* 129:55–62
111. Rosenblatt, S., Chanley, J. D. 1965. *Arch. Gen. Psychiat.* 13:495–502
112. Rosenblatt, S., Chanley, J. D., Leighton, W. P. 1969. *J. Psychiat. Res.* 6:321–33
113. Murphy, D. L., Wyatt, R. J. 1972. *Nature* 238:225–26
114. Wyatt, R. J. et al 1973. *Science* 173: 916–18
115. Nies, A., Robinson, D. S., Lamborn, K. R., Lampert, R. P. 1973. *Arch. Gen. Psychiat.* 28:834–38

116. Carlson, G. A., Goodwin, F. K. 1973. *Arch. Gen. Psychiat.* 28:221–28
117. Carpenter, W. T. Jr., Strauss, J. S., Muleh, S. 1973. *Arch. Gen. Psychiat.* 28:847–52
118. Shih, J.H.C., Eiduson, S. 1971. In *Brain Chemistry and Mental Disease,* ed. B. T. Ho, W. M. Mc Isaac, 3–20. New York: Plenum
119. Kety, S. S. 1971. See Ref. 118, pp. 237–44

Copyright 1974. All rights reserved

VITAMIN D METABOLISM

◆7118

M. F. Holick, Ph.D. and H. F. DeLuca, Ph.D.
Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin

INTRODUCTION

The recent introduction of new techniques in organic synthesis, chromatography, and structural identification has brought forth a rebirth of interest and exciting new discoveries with medical importance in the area of vitamin D metabolism and function. In 1963–1966 the synthesis of radioactive vitamin D of very high specific activity was completed, permitting experiments with physiologic doses. In addition, separation methods common to the study of lipid metabolism were applied to a study of vitamin D metabolism. In 1968 the tools of mass spectrometry, nuclear magnetic resonance spectroscopy, and other modern techniques of chemical identification were introduced with the resulting identification of vitamin D metabolites. In addition, the full realization of multiple functions of vitamin D has allowed a more thorough understanding of the interrelationship between vitamin D and parathyroid hormone in the regulation of calcium and phosphate metabolism. It is to these specific areas that this review will be addressed. However, space limitation prevents consideration of many excellent investigations of calcium and phosphorus metabolism related to vitamin D.

PHYSIOLOGICAL FUNCTIONS OF VITAMIN D

From the original experiments of Mellanby, McCollum, Huldshinsky, Hess, and Steenbock (1–6), it is evident that vitamin D is in some way involved in the maintenance of healthy bone. Although it has been intellectually satisfying to consider that vitamin D must function directly on bone in the mineralization process, evidence to support this idea is lacking. Instead, the weight of evidence is contrary to this idea and it appears that the primary function of vitamin D in promoting calcification is the elevation of plasma calcium and phosphate to supersaturating levels in the plasma and extracellular fluid. A deficiency of vitamin D results in a subnormal level of either plasma calcium or phosphate (7, 8) or both.

Although the depressed plasma calcium and phosphorus product in rickets was recognized by Howland & Kramer in 1921 (7), it was not until 1937 that Nicolaysen (9, 10) convincingly demonstrated that a major function of vitamin D is the stimulation of intestinal calcium absorption. This process is an active one requiring metabolic energy to transport calcium from the lumen of intestine to the serosal fluid. It is maximal in the duodenum, followed by jejunum, ileum, and is even present in the colon (11, 12). The calcium transport system has been thoroughly reviewed (13–16).

Another important function of vitamin D is to stimulate the mobilization of calcium from bone. This was first demonstrated in 1952 by Carlsson (17) and was later shown by Harrison & Harrison (18) and Rasmussen et al (19) to require the participation of both vitamin D and parathyroid hormone. The biochemistry of this system is not at all understood but it is of great importance in the maintenance of serum calcium concentration and must be viewed as another mechanism whereby vitamin D increases serum calcium and phosphorus concentrations.

In 1941 Harrison & Harrison suggested that vitamin D increases renal tubular reabsorption of phosphorus (20). Although there have been many reservations put forth about the initial observations, there is still adequate reason to believe that vitamin D stimulates renal tubular reabsorption of phosphorus, calcium, and possibly sodium (21–23). However, much remains to be done in establishing this important function. It seems likely that these three basic functions of vitamin D in intestine, bone, and kidney contribute to elevating plasma calcium and phosphorus concentrations to levels sufficient for normal mineralization to occur. How vitamin D is metabolized to carry out these functions is the major subject of the remaining review.

VITAMIN D METABOLISM

25-Hydroxyvitamin D_3

The first metabolic investigations must be accredited to Kodicek who demonstrated with laborious biological assay techniques that vitamin D is metabolized to biologically inactive products (24). He and his co-workers prepared radioactive vitamin D_2 of low specific activity and were unable to detect metabolites of vitamin D with biological activity (25, 26) because they were forced to use pharmacological doses. Vitamin D_3 of moderate to high specific activity was prepared in 1963–1966 (27, 28). As a result, Lund & DeLuca, using physiological amounts of radioactive vitamin D_3 of high specific activity, demonstrated the existence of biologically active metabolites (29). The most polar metabolite was effective in the cure of rickets in rats and it acted more rapidly than vitamin D in initiating intestinal calcium transport (30). This metabolite was isolated in pure form from porcine plasma (31) and later from human plasma (32) and identified as 25-hydroxyvitamin D_3 (25-OH-D_3) (Figure 1). The major site for the 25-hydroxylation is the liver (33–35).

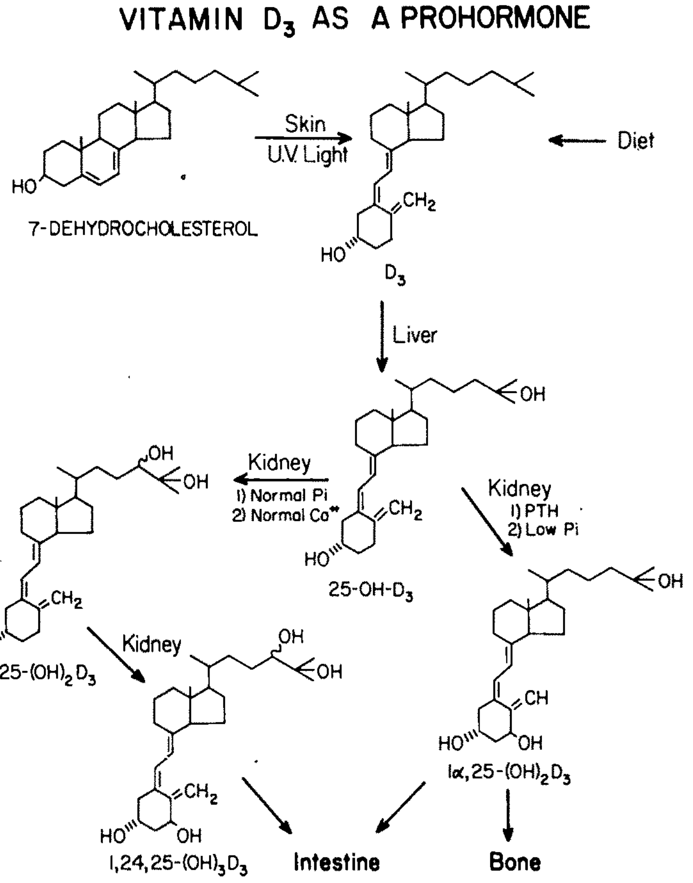


Figure 1 Schematic summary of the origin of vitamin D$_3$ and its various biologically active metabolities.

25,26-(OH)$_2$D$_3$ and 24,25-(OH)$_2$D$_3$

Although at first it was thought that 25-OH-D$_3$ is the biologically active form of the vitamin (36), it became apparent that there are metabolites of vitamin D$_3$ more polar than 25-OH-D$_3$ that possess biological activity. When pigs are fed large amounts of vitamin D$_3$ (2.5 mg/day in the diet), there are at least seven metabolites more polar than 25-OH-D$_3$ in their plasma (37, 38). To date two of these metabolites have been isolated in pure form and identified as 25,26-dihydroxyvitamin D$_3$ (38) [25,26-(OH)$_2$D$_3$] and 21,25-dihydroxyvitamin D$_3$ [21,25-(OH)$_2$D$_3$] (37). A more complete identification of the latter metabolite proved it to be 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] (39) (Figure 1). Similarly it has been demonstrated that in a hypoparathyroid patient who received 0.25 mg vitamin D_3/day, one of the major circulating metabolites is 24,25-$(OH)_2D_3$ (32).

1,25-$(OH)_2D_3$

Of major interest, however, is a more polar metabolite of vitamin D_3 and 25-OH-D_3 which appears in the target tissues, namely the intestine and bone, of vitamin D-deficient rats and chickens that receive [1,2-^{3}H]-vitamin D_3 or [25,26-^{3}H]-25-OH-D_3 (40–44).

Haussler et al were the first to demonstrate that this metabolite from the intestine of chicks given [1,2-^{3}H]-vitamin D_3 stimulates intestinal calcium transport (40). This metabolite acts more rapidly than 25-OH-D_3 in initiating intestinal calcium transport and bone calcium mobilization (45–48). In 1971 two laboratories simultaneously reported the structural identification of this metabolite. Holick et al (49, 50) isolated 2 μg of this metabolite in pure form from the intestines of 1500 chickens and identified it as 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (Figure 1), while Lawson et al (51) isolated the metabolite from chicken kidney homogenates and provided evidence for the same structure.

Role of the Kidney in Vitamin D Metabolism

Even before its identification, Fraser & Kodicek (52) made the important observation that 1,25-$(OH)_2D_3$ is made exclusively in the kidney from 25-OH-D_3 (Figure 1). This result was quickly confirmed and extended to demonstrate that it was not uremia resulting from the nephrectomy that prevents this conversion but rather the lack of kidney tissue per se (53, 54). Furthermore, in the absence of kidneys, 25-OH-D_3 is unable to induce either intestinal calcium transport or bone calcium mobilization. However, 1,25-$(OH)_2D_3$ is fully effective in initiating both intestinal calcium transport and bone calcium mobilization in anephric animals (55–57). These results made possible the conclusions that 1,25-$(OH)_2D_3$ or a further metabolite and not 25-OH-D_3 is the metabolically active form of vitamin D responsible for initiating intestinal calcium transport and bone calcium mobilization. Further it could be ascertained that bone disease associated with renal failure might be at least in part due to a failure of renal synthesis of 1,25-$(OH)_2D_3$.

Vitamin D_3 Hydroxylation Reactions In Vitro

Although the 25-hydroxylation reaction has been studied in homogenates and subcellular fractions of animals, it has not yet been demonstrated in tissues of man. In animals, the 25-hydroxylation reaction occurs primarily in the endoplasmic reticulum of liver and requires NADPH and molecular oxygen. It does not appear to be a cytochrome P-450 dependent reaction (58). The 25-OH-D-1-hydroxylase occurs in kidney mitochondria, is dependent upon NADPH and molecular oxygen, and in all respects appears to be a cytochrome P-450 reaction (52, 59, 60). The 25-OH-D_3-

24-hydroxylase is also found in the renal mitochondria, it likewise is dependent upon a source of NADPH and molecular oxygen, but does not appear to be blocked by inhibitors that block cytochrome P-450 reactions (61).

Metabolism of Vitamin D in Man

The development of methods to study metabolism of vitamin D in animals led naturally to its application in man. Avioli and his colleagues pioneered the investigations of tritiated vitamin D_3 in man (62, 63). From those early investigations it became clear that vitamin D is metabolized to polar metabolites in man as well as in animals. However, the early work with tritiated vitamin D_3 must be regarded with great caution, as has been pointed out by Stanbury and his colleagues (64). Comparison of metabolism of tritiated vitamin D_3 in normal man to those suffering from bone disease or those that had been treated with large doses of some form of vitamin D is not possible by simply comparing disappearance of radioactivity from blood or appearance of peaks of radioactivity in chromatographic profiles (65).

From all of the tritiated vitamin D studies in man that have been published it can be stated only that vitamin D is rapidly cleared from the plasma, probably is taken up in the liver initially, and then redistributed in a variety of tissues, especially in fat depots (65). This agrees with experiments carried out on the storage of vitamin D in animals by Rosenstreich and co-workers (66). The primary excretory route is in the bile and only about 2% of the radioactivity appears in the urine (62, 65). The nature of the metabolites appearing in bile and urine remain unknown, although it seems clear that they are primarily polar metabolites conjugated with glucuronic acid or sulfate (67).

The only metabolites that have been positively identified in the plasma of man are 25-OH-D_3 and 24,25-$(OH)_2D_3$ (32). Mawer et al and Shaefer et al (68, 69) have provided evidence that 1,25-$(OH)_2D_3$ is formed in man.

There have been successful attempts at development of assays for 25-OH-D_3 and vitamin D_3, using competitive protein binding. Both Belsey et al (70, 71) and Haddad and co-workers (72) appear to have developed excellent assays capable of yielding important new information on plasma vitamin D and 25-OH-D_3 levels in disease. Furthermore, they provide a method for estimating pool sizes that can then be used together with tritiated vitamin D and 25-OH-D_3 to study metabolism in man more meaningfully. Haddad and co-workers have successfully applied his method in the measurement of 25-OH-D_3 in a variety of circumstances. Using this technique they have been able to demonstrate that there is a markedly decreased level of 25-OH-D_3 in the serum of patients on anticonvulsant therapy (73) but that the level of this metabolite remains normal under conditions of sex-linked hypophosphatemic vitamin D-resistant rickets (74). Undoubtedly application of such methods in the future will lead to sound information relating disease states to vitamin D metabolism.

Vitamin D Transport Proteins

Although there is insufficient space to discuss all aspects of vitamin D metabolism in man, it is important to realize that there are specific proteins in the blood that are charged with the responsibility of transporting vitamin D and 25-OH-D_3 to and from target tissues. Specific transport proteins for vitamin D were demonstrated by Chalk & Kodicek (75), and were more firmly established by the work of Rikkers & DeLuca (76) and Crousaz et al (77). The 25-OH-D_3 binding protein has been isolated in relatively pure form and studied by Peterson (78).

Metabolism of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$

Although 1,25-$(OH)_2D_3$ can act in anephric rats while 25-OH-D_3 cannot, it is still conceivable that 1,25-$(OH)_2D_3$ might require further metabolic alteration before it can stimulate intestine and bone. Tritiated 1,25-$(OH)_2D_3$ was injected at physiological doses into both chicks and rats (79–81). When the intestine was transporting calcium maximally in response to the ^3H-1,25-$(OH)_2D_3$, more than 80% of the radioactivity in intestine and bone was present as unchanged 1,25-$(OH)_2D_3$. No other metabolites were detected at that time. Similarly, it was noted that at 6 and 12 hr after dosing, when bone calcium mobilization was maximally stimulated in the rat, the only detectable metabolite in the bone was unaltered 1,25-$(OH)_2D_3$. These results establish 1,25-$(OH)_2D_3$ as a metabolically active form of vitamin D_3 in these two target organs.

Recently Boyle et al (82, 83) demonstrated that under normal or hypercalcemic conditions the major circulating metabolite of 25-OH-D_3 is 24,25-$(OH)_2D_3$. This metabolite, which is synthesized in the kidney (Figure 1), is capable of supporting growth, elevating serum calcium, and calcifying bones of rats on a normal calcium, normal phosphorus diet (82). Their data demonstrate that 24,25-$(OH)_2D_3$ is capable of inducing intestinal calcium transport at dose levels similar to 1,25-$(OH)_2D_3$ but has little ability to mobilize calcium from the bone. 24,25-$(OH)_2D_3$ is metabolized to a more polar metabolite by the kidney, which appears necessary for it to bring about these biological responses. This polar metabolite has been prepared from 24,25-$(OH)_2D_3$ biosynthetically with chicken kidney homogenates. It has been isolated in pure form and identified as 1,24,25-trihydroxyvitamin D_3 [1,24,25-$(OH)_3D_3$] (84) (Figure 1). This metabolite stimulates intestinal calcium transport but has little activity in mobilizing calcium from the bone. It is about 60% as effective as vitamin D_3 in the cure of rachitic lesions and, on a weight basis, it is about two thirds as active as 1,25-$(OH)_2D_3$ in initiating intestinal calcium transport in rats. Unfortunately, the biological significance of the 1,24,25-$(OH)_3D_3$ is unknown and the possibility exists that it may represent a minor and insignificant pathway of vitamin D metabolism.

Regulation of Vitamin D Metabolism

REGULATION OF VITAMIN D-25-HYDROXYLATION Vitamin D_3-25-hydroxylase, found in the endoplasmic reticulum of liver, is subject to a degree of feedback regulation (58). This enzymatic activity is highest in vitamin D-deficient animals and hence is probably not inducible. Administration to animals of radioactive or nonradioactive vitamin D_3 results within 3 hr in a marked depression of the 25-hydroxylase activity of liver homogenates and isolated microsomes. The degree and length of this depressed 25-hydroxylase activity is related to the dose of the vitamin given. The larger the dose, the more rapid the reduction in the hydroxylase activity, the greater the extent, and the greater the length of time before the hydroxylase activity recovers. The exact nature of the regulation is not yet understood. Dihydrotachysterol$_3$ (DHT$_3$) (Figure 2), a close analog of vitamin D_3, is also 25-hydroxylated by the liver, but this hydroxylation takes place both in mitochondria and microsomes, and is unaffected by predosing of animals with either vitamin D_3 or DHT$_3$ (85). The regulation of vitamin D_3-25-hydroxylation has been verified in vivo by the demonstration that the rate of appearance of 25-OH-D_3 in the blood from vitamin D_3 is not linearly related to the dose of vitamin D given. Instead there appears a plateau at relatively low doses of vitamin D_3, beyond which there is only a 2–3-fold increase of blood 25-OH-D_3 with every 1000-fold increase in vitamin D_3 dosage. In contrast, 25-hydroxydihydrotachysterol$_3$ (25-OH-DHT$_3$) (Figure 2) increases in the blood linearly with respect to the dose of DHT$_3$ given, revealing that this analog escapes a control mechanism to which vitamin D_3 itself is subject. This may account in part for its greater effectiveness than vitamin D at high doses. It must be emphasized, however, that the regulation of vitamin D_3-25-hydroxylation can be overcome inasmuch as it is possible to increase blood levels of 25-OH-D_3 by greatly increasing the dosage of vitamin D (31).

Because 25-OH-D_3 but not vitamin D_3 has a direct effect on mobilization of calcium from bone (86), it seems possible that at least part of the toxicity due to vitamin D may be due to the elevated levels of 25-OH-D_3 found in the plasma under conditions of hypervitaminosis D. Vitamin D toxicity can be experienced in completely nephrectomized animals (87), which would suggest that the 1,25-$(OH)_2D_3$, which is made exclusively in kidney, is not necessary for toxicity. The 25-hydroxylation mechanism and its regulation must therefore be viewed as a bottleneck reaction that provides the first regulatory step in the function of vitamin D.

REGULATION OF THE 1- AND 24-HYDROXYLASES OF 25-OH-D_3 IN THE KIDNEY The realization that 1,25-$(OH)_2D_3$ is a metabolically active form of the vitamin in both intestine and bone gave birth to the idea that dietary and serum calcium might regulate its synthesis (83). It had long been known from the work of Nicolaysen et al (88), Rottensten (89), and Fairbanks & Mitchell (90) that animals given vitamin D show a marked ability to adapt to dietary calcium. Those on low calcium diets adapt by showing a markedly elevated intestinal calcium absorption while those on a high calcium diet show a markedly reduced efficiency of calcium absorption. In both chicks and rats supplemented with small amounts of vitamin D it is clear that low calcium diets result in marked synthesis of 1,25-$(OH)_2D_3$, while diets containing adequate or high levels of calcium result in a depressed synthesis of 1,25-$(OH)_2D_3$ (83, 91). In an almost reciprocal fashion rats on a high calcium diet show marked production of 24,25-$(OH)_2D_3$, while those on a low calcium diet show virtually no formation of this metabolite. Vitamin D-deficient animals, regardless of dietary calcium and phosphorus levels, make only 1,25-$(OH)_2D_3$ when challenged with a single pulse dose of radioactive 25-OH-D_3. Thus, adaptation or regulation of the hydroxylases of kidney takes place only in animals receiving a source of vitamin D and is not readily apparent in vitamin D-deficient animals.

that the regulation point of intestinal calcium adaptation is likely at the level of 1,25-$(OH)_2D_3$ formation (16).

In intact animals on diets adequate in phosphorus and containing vitamin D, the regulation of 1,25-$(OH)_2D_3$ formation is related to serum calcium concentration. Under conditions of hypocalcemia, 1,25-$(OH)_2D_3$ is produced in large amounts, whereas under conditions of normal or hypercalcemia its production is markedly reduced. The 24,25-$(OH)_2D_3$ shows an exact reverse regulation, namely that under conditions of hypocalcemia no 24,25-$(OH)_2D_3$ is made, whereas under conditions of normal and hypercalcemia this metabolite is preferentially formed. There is now strong evidence that this regulation of vitamin D metabolism under physiologic conditions is under control of the parathyroid glands (95–98). It seems likely that secretion of parathyroid hormone in response to hypocalcemia in some manner stimulates the production of 1,25-$(OH)_2D_3$. Hypercalcemia, which results in depressed parathyroid secretion, shuts down 1,25-$(OH)_2D_3$ production. This important relationship not only brings together another point of interaction between the polypeptide hormone and vitamin D, but raises an important question of the relative importance of this interaction in calcium homeostasis.

A study of the response of vitamin D-deficient animals that are thyroparathyroidectomized to 1,25-$(OH)_2D_3$, both in the presence and absence of the exogenous parathyroid hormone, reveals that the intestinal calcium transport response to this hormonal form of vitamin D does not require the presence of parathyroid hormone whereas the mobilization of calcium from bone requires both 1,25-$(OH)_2D_3$ and the parathyroid hormone (Garabedian and DeLuca, unpublished results). It therefore seems important to realize that a new aspect of calcium homeostasis is the stimulation of 1,25-$(OH)_2D_3$ formation in the kidney by parathyroid hormone, which in turn brings about mobilization of calcium from bone and intestine (Figure 3).

The role of calcitonin, if any, in the regulation of the 25-OH-D_3 kidney hydroxylases is not at all understood at the present time. Some evidence for regulation has been offered by Galante et al (99), who demonstrated that injection of sizable amounts of calcitonin might actually stimulate production of 1,25-$(OH)_2D_3$. A similar protocol involving parathyroid hormone suggested that this hormone might depress production of 1,25-$(OH)_2D_3$ (100). Although these findings are of potential significance in unraveling the molecular mechanism of the regulation of the 25-OH-D_3 hydroxylases in the kidney, it seems difficult to place them in their proper physiologic perspective.

Hypophosphatemia induced by dietary means also stimulates production of 1,25-$(OH)_2D_3$, even in the absence of the parathyroid glands (101). In fact, if the thyroid and parathyroid glands are removed, there is a close relationship between serum inorganic phosphorus and the ability of animals to produce 1,25-$(OH)_2D_3$. Thus thyroparathyroidectomized animals that are hypophosphatemic produce sizable quantities of 1,25-$(OH)_2D_3$, whereas when they are hyperphosphatemic they produce 24,25-$(OH)_2D_3$. It is therefore evident that inorganic phosphate levels also play an important regulatory role with regard to the 24-OH-D_3 kidney hydroxylases.

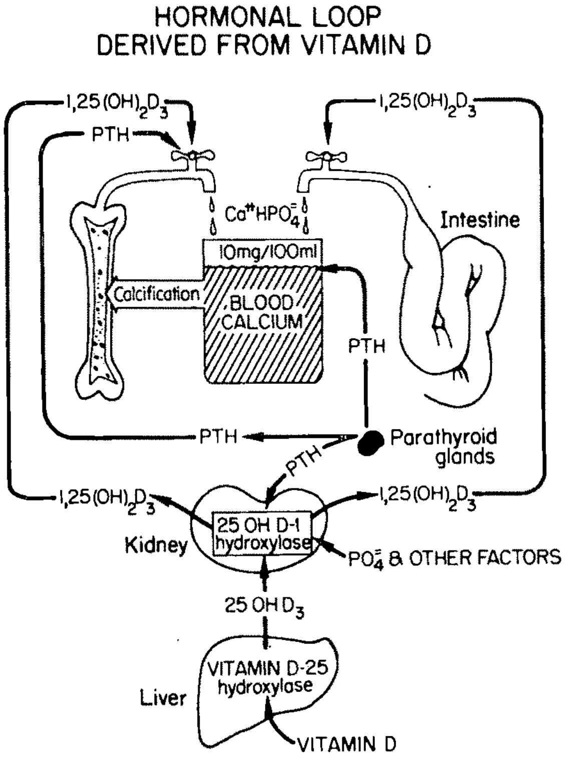

HORMONAL LOOP
DERIVED FROM VITAMIN D

Figure 3 Schematic representation of the hormonal control loop for vitamin D metabolism and function. A reduction in the serum calcium from 10 mg/100 ml serum prompts a proportional secretion of parathyroid hormone that acts to mobilize calcium from the bone. Parathyroid hormone also stimulates the synthesis of $1,25\text{-(OH)}_2D_3$ in kidney, which in turn stimulates the mobilization of calcium from bone and intestinal contents. As serum calcium increases to towards normal (10 mg/100 ml serum), parathyroid secretion is proportionately decreased.

Chicks on low phosphorus diets show markedly increased ability to absorb calcium (102). It has now been demonstrated that this increased ability can be directly related to increased production of $1,25\text{-(OH)}_2D_3$ and its appearance in the intestine (103). Therefore, both parathyroid hormone and hypophosphatemia can be regarded as stimulatory agents for the production of this hormone derived from vitamin D. It has also been suggested that parathyroid hormone may stimulate the production of $1,25\text{-(OH)}_2D_3$ by inhibiting tubular reabsorption of inorganic phosphorus and that it is the renal cell level of inorganic phosphorus which in some way regulates production of $1,25\text{-(OH)}_2D_3$ (101). Although there is some evidence for this interesting possibility, considerable work will have to be done before this can be established.

The molecular mechanism of regulation of $1,25\text{-}(OH)_2D_3$ production is not yet understood. It is clear that the regulation of the $1,25\text{-}(OH)_2D_3$ is a relatively slow mechanism requiring hours if not days for complete changeover from 1-hydroxylation to 24-hydroxylation and vice versa (95). Experiments involving changes of dietary calcium and phosphate show that there are associated rapid changes in serum calcium and phosphate concentrations yet the change in hydroxylation takes place over a period of days (Boyle, Gray & DeLuca, unpublished results). Because the estimated half-life of the $25\text{-}OH\text{-}D_3$-1-hydroxylase is 2–4 hr (104), changes in hydroxylation might well involve enzyme synthesis and/or degradation. Possibly renal tubule cell levels of either calcium or phosphorus or both, as well as the presence of vitamin D metabolites, interact in a complex manner to determine which of the hydroxylases is produced and which is degraded.

From the studies thus far on the regulation of the $1,25\text{-}(OH)_2D_3$-producing system of the kidney have come at least two possible medically important facts. One is that hypoparathyroid patients may have limited ability to produce $1,25\text{-}(OH)_2D_3$ and that they therefore should be managed with relatively low doses of either $1,25\text{-}(OH)_2D_3$ or its close analog $1\alpha\text{-}OH\text{-}D_3$ plus dietary calcium. It seems likely that in this disease the bone calcium mobilization system will not operate normally and must be replaced entirely by the intestinal calcium absorption mechanism for maintaining serum calcium concentration. Another important possible medical application is the realization that hypophosphatemia stimulates production of $1,25\text{-}(OH)_2D_3$. It therefore seems important in the management of both hypoparathyroid patients and patients suffering from chronic renal disease to restrict dietary phosphorus not only from the point of view of preventing unwanted soft tissue mineralization, but also to stimulate production of $1,25\text{-}(OH)_2D_3$ by residual 1-hydroxylase enzyme which remains in the diseased tissue or in the hypoparathyroid tissue. It is also possible that an early event in chronic renal disease may be an alteration in phosphate and calcium concentrations of the renal tubule cells resulting in a turning off of $1,25\text{-}(OH)_2D_3$ synthesis, which may contribute or play a major role in the chain of events which result in renal osteodystrophy.

DISTURBANCE OF CALCIUM METABOLISM DUE TO ANTICONVULSANT DRUGS

In 1968 Kruse (105) first suggested a relationship between the appearance of rickets and osteomalacia in children and long-term antiepileptic therapy. Two years later, Richens & Rowe (106) and Dent and associates (107) demonstrated an association of hypocalcemia with high dosages of anticonvulsant drugs. Individual anticonvulsant drugs produced hypocalcemia in the following order: pheneturide, primidone, phenylhydantoin, and phenobarbitone. They concluded that osteomalacia and rickets in children with epilepsy was related to the number and dose of anticonvulsant drugs as well as the duration of therapy.

Kruse found low levels of vitamin D_3 in the serum of two of his patients on antiepileptic therapy. It has been suggested but not established that the basic reason for altered calcium metabolism due to anticonvulsant therapy is that the drugs induce a large number of hydroxylases in the liver endoplasmic reticulum, which converts vitamin D_3 to inactive metabolites (108, 109). In support of this theory, Stamp et al (73) have shown reduced levels of 25-OH-D_3 in the plasma of patients on antiepileptic drugs. Furthermore, they can correct the bone disease with small doses of 25-OH-D_3. Hunter et al (110) have found that almost all children receiving antiepileptic drugs excrete excessive amounts of D-gluconic acid in the urine, indicative of hepatic enzyme induction. More recent studies have demonstrated that 25-OH-D_3 is also rapidly metabolized to apparently biologically inactive polar metabolites in patients receiving chronic therapy with either phenobarbital or diphenylhydantoin (111).

Treatment of this drug-induced disease has varied. Kruse (105) has reported that 400–800 units of vitamin D_3 administered orally per day is sufficient for treating many of his patients. Others have reported the need to increase the dosage from 2000 units daily to 50,000 units before a significant rise in serum calcium and phosphorus and a decrease in serum alkaline phosphatase can be observed. Furthermore, Hahn and associates (111, 112) suggested that it is prudent to provide vitamin D supplementation in excess of 4000 units of vitamin D per week to the average adult outpatient epileptic on the usual clinical doses of phenobarbital and diphenylhydantoin to maintain normal serum 25-OH-D_3 levels.

THE EFFECT OF ADRENAL HORMONES ON VITAMIN D-MEDIATED INTESTINAL CALCIUM TRANSPORT

It was originally observed by Harrison & Harrison in 1960 (113) that cortisol treatment reduced the rate of vitamin D-mediated intestinal calcium absorption. It therefore appeared that cortisol treatment antagonized and partially inhibited the action of vitamin D in its ability to stimulate intestinal calcium transport. Similar results were obtained in the chicken after the administration of cortisol or deoxycorticosterone (114, 115). These experiments provided a rationale for the clinical practice of treating hypercalcemia and hypercalciuria related to sarcoidosis, nephrocalcinosis, and vitamin D intoxication with cortisone in both infants and adults (116–118).

Although clinical experience and animal experiments indicate that glucocorticoids are antagonistic to the action of vitamin D, the basis for this apparent antagonism remains uncertain. One possibility, namely alteration in vitamin D_3 metabolism, has been investigated. Avioli and co-workers (119) found that during prednisone administration there was a decrease in the amount of vitamin D_3 and 25-OH-D_3 found in the plasma in comparison to normal controls. They, therefore, suggested that glucocorticoids are similar to the anticonvulsant drugs in that they induce liver hydroxylase enzymes, which modify vitamin D in such a way as to render it inactive. However, more recent experiments in rats suggest that the cortisone effect in the intestine is not necessarily due to a defect in the metabolism of vitamin D to 25-OH-D_3. Kimberg et al (120) have shown that cortisone also alters the transport of iron and galactose, which are not dependent on vitamin D, thus suggesting that cortisone treatment may be responsible for more than a simple antagonism to the effects of vitamin D.

CLINICAL USE OF 25-OH-D_3

25-OH-D_3 was found in animal experiments to initiate intestinal calcium transport and bone calcium mobilization more rapidly than vitamin D_3, and on a weight basis is 1.5–5 times more effective than the parent vitamin in the cure of rickets in rats (121). As a result, 25-OH-D_3 was enthusiastically received by clinicians in the hope that diseases such as vitamin D-resistant rickets and osteodystrophy caused by chronic renal failure may be due to a defect in the metabolism of vitamin D to 25-OH-D_3. Although 25-OH-D_3 is extremely effective in promoting rapid remineralization of skeleton in human deficiency rickets when given at a dosage as low as 2 μg/day for two months, this efficacy relative to vitamin D_3 is not increased in the treatment of the various vitamin D-resistant syndromes (122). At the same dosage provided in vitamin D-deficiency rickets, 25-OH-D_3 was ineffective in the treatment of familial hypophosphatemic vitamin D-resistant rickets, pseudodeficiency rickets, or rickets secondary to cystinosis and tyrosinosis (122, 123). Resistance to vitamin D in idiopathic or acquired hypoparathyroidism, although uncommon, has also been treated successfully with pharmacological amounts of 25-OH-D_3 (124). As with vitamin D_3 and DHT_3, patients vary greatly in the doses of 25-OH-D_3 to which they respond. Some patients respond favorably to 0.1 mg (5000 units) or less while many others respond poorly to doses as high as 1 mg (50,000 units). Nevertheless, the use of 25-OH-D_3 in bone disease represents a marked advance over the use of vitamin D_2 and vitamin D_3 because of its greater effectiveness and shorter lifetime in the body.

CLINICAL USES OF 1,25-$(OH)_2D_3$

With the revelation that 25-OH-D_3 is unable to stimulate either intestinal calcium transport or bone calcium mobilization until it is hydroxylated at the C-1 position in the kidney, it became obvious why 25-OH-D_3 was not as useful as anticipated in the management of at least some vitamin D-resistant diseases. Attention has now been focused on the potential use of 1,25-$(OH)_2D_3$ in the treatment of patients with hypocalcemia, impaired intestinal calcium transport, secondary hypoparathyroidism, and osteodystrophy related to renal dysfunction. Brickman and co-workers (125) were the first to demonstrate the efficacy of 1,25-$(OH)_2D_3$ in three patients with advanced renal failure and with one anephric patient on regular dialysis. Each patient received 2.7 μg of 1,25-$(OH)_2D_3$ daily for 6–10 days. In each case there was a significant increase in intestinal calcium absorption above the pretreatment values and the intestinal absorption was associated with an increase in the serum calcium concentration. Furthermore, in two of the patients, metabolic balance studies showed a decrease in fecal calcium excretion and improvement in the calcium balance.

Recently 1,25-$(OH)_2D_3$ has been successfully used at physiologic doses in the management of vitamin D-dependent rickets, a recessively inherited form of vitamin D-refractory rickets characterized by hypocalcemia, hypophosphatemia, elevated

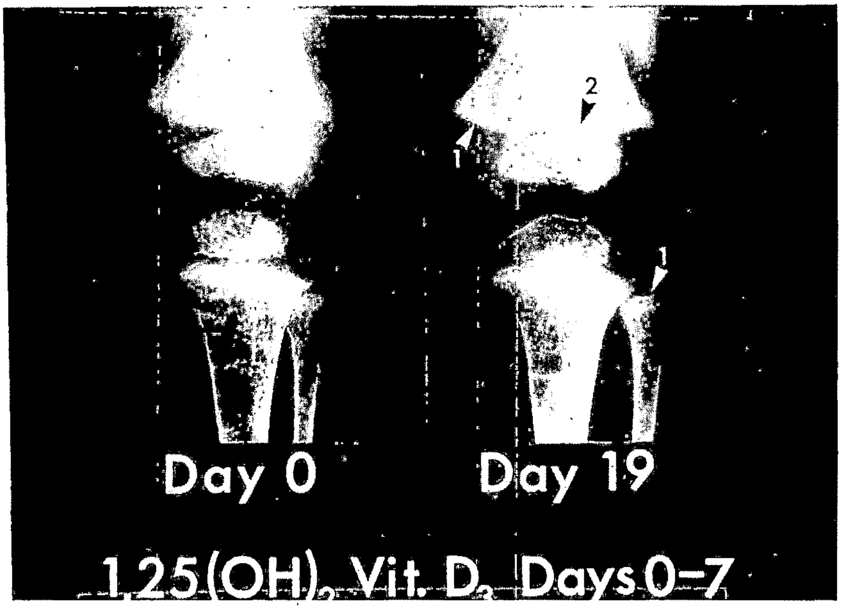


Figure 4 Radiograph of left knee of a patient with active vitamin D-dependency rickets (day 0) and 19 days later after 7 days of intravenous therapy with 1 μg of 1 α,25-$(OH)_2D_3$ per day. Arrows (1) indicate new mineral deposits and arrow (2) indicates narrowing of the space between the metaphysis and epiphysis.

serum alkaline phosphatase activity, and severe rachitic lesions (126). Fraser et al observed that although large amounts of vitamin D_2 (1.25–2.5 mg/day), vitamin D_3 (1.25 mg/day), and 25-OH-D_3 (0.4–0.9 mg/day) were capable of healing rachitic lesions, only very small doses of 1α,25-$(OH)_2D_3$ (1 μg/day) were needed to initiate healing (Figure 4). These observations prompted these investigators to speculate that vitamin D-dependent rickets is an inborn error of metabolism caused by the block in the conversion of 25-OH-D_3 to 1,25-$(OH)_2D_3$.

The results in the case of these two disease states strongly suggest that 1,25-$(OH)_2D_3$ will be useful in the management of a number of calcium metabolism disorders, especially in those related to renal insufficiency disease. However, the report by Glorieux et al (127) that 1 μg/day of 1,25-$(OH)_2D_3$ is ineffective in healing rickets or elevating serum phosphorus in X-linked hypophosphatemic patients demonstrates that 1,25-$(OH)_2D_3$ will also have its limitations in treatment of vitamin D-resistant bone disease.

BIOLOGICALLY ACTIVE ANALOGS OF VITAMIN D

A number of analogs of vitamin D metabolites with interesting biological properties have been prepared. Dihydrotachysterol (AT-10) (Figure 2) has been known to the medical world for many years. Although this analog has very little antirachitic activity, it is capable of raising serum calcium concentrations in parathyroidectomized rats (128) and in hypoparathyroid patients. In 1970 Suda et al (129) synthesized the 25-hydroxy derivative of DHT_3 (Figure 2) and demonstrated on a weight basis that it is biologically more active than DHT_3. Recently it has been suggested that the main reason that DHT_3 and 25-OH-DHT_3 can function in situations where vitamin D is refractory is because this compound is an analog of 1,25-$(OH)_2D_3$. The rotated A-ring in the DHT_3 and 25-OH-DHT_3 structure provides the 3β-hydroxyl in a position geometrically similar to the 1α-hydroxyl of 1,25-$(OH)_2D_3$ (130). Thus it is not surprising that both 25-OH-DHT_3 and DHT_3 are capable of stimulating intestinal calcium transport and bone calcium mobilization in anephric rats, hypoparathyroid rats, and in patients suffering from renal failure (131).

Four analogs of 1,25-$(OH)_2D_3$, which can be simply synthesized from vitamin D_3 or 25-OH-D_3, have been prepared and their biological activity studied (132–134). The 5,6-transvitamin D_3, isotachysterol$_3$, 5,6-trans-25-OH-D_3, and 25-hydroxyisotachysterol$_3$ (Figure 2) have their A-ring rotated relative to vitamin D_3 such that the 3β-hydroxyl becomes spatially located in the geometric position occupied by the 1α-hydroxy of 1,25-$(OH)_2D_3$ (Figure 2). These analogs are all capable of stimulating intestinal calcium transport in anephric rats, and in addition the 5,6-transvitamin D_3, isotachysterol$_3$, and 25-hydroxyisotachysterol$_3$ can also stimulate bone calcium mobilization in these animals. Surprisingly the 5,6-trans-25-OH-D_3 has little ability to stimulate bone calcium mobilization. This peculiar property of the 5,6-trans-25-OH-D_3 suggests its possible use in the management of chronic renal failure since it would be desirable to stimulate intestinal calcium transport without stimulating bone calcium mobilization.

Although it is possible that these analogs may be useful in the treatment of various vitamin D-refractory syndromes, one must note that these analogs are about 100–1000 times less active on a weight basis than 1,25-$(OH)_2D_3$. For example, Steele et al (135) and Rutherford et al (136) have reported that dosages of 0.1–5 mg of 5,6-trans-D_3 were needed to correct calcium malabsorption in anephric patients and patients with chronic renal disease. In contrast, however, 1,25-$(OH)_2D_3$ produced similar results at a dosage of 1 μg/day (135).

The most promising analog of 1,25-$(OH)_2D_3$ is 1α-OH-D_3 (Figure 2). This analog is much less difficult to prepare chemically than 1α,25-$(OH)_2D_3$ (137) and is much less expensive to make in large quantities. Unlike the other 1α-hydroxy analogs just discussed, which are at least 100 times less active than 1,25-$(OH)_2D_3$, 1α-OH-D_3 is as biologically active on a weight basis as 1,25-$(OH)_2D_3$ in promoting intestinal calcium transport, bone calcium mobilization, and in its ability to heal rachitic lesions (138). Furthermore, 1α-OH-D_3 has the advantage of being equally as active when given orally, intraperitoneally, or intravenously, while 1,25-$(OH)_2D_3$ is apparently not very effective when given by the oral route, especially in an oil solution. The 1α-OH-D_3 has already been successfully applied in the management of serum calcium in a vitamin D-resistant hypoparathyroid patient (Gegick, Holick & DeLuca, unpublished results). Before treatment this patient had a serum calcium of 5–6 mg% and was resistant to over 12.5 mg (600,000 units) of vitamin D_3/day. Upon treatment with 1 μg 1α-OH-D_3 orally, together with 2 g calcium per day, the serum calcium rose to normal within two days. It seems likely that this analog of 1,25-$(OH)_2D_3$ will be the drug of choice in the treatment of disease that is associated with defective 1-hydroxylation of vitamin D_3 such as renal osteodystrophy, hypoparathyroidism, and vitamin D-dependency disease. It would appear that the physicians, armed with 25-OH-D_3 for cases of defective 25-hydroxylation on one hand and 1α-OH-D_3 on the other, will be prepared to manage many diseases of calcium or phosphorus metabolism that may be related to defective vitamin D metabolism.

SUMMARY

There have been great advances in our understanding of the metabolism of vitamin D in experimental animals. It can be demonstrated that vitamin D_3 first must be hydroxylated on carbon 25 in the liver and subsequently on carbon 1 in the kidney before it can stimulate intestinal calcium absorption and bone calcium mobilization. The resultant 1,25-$(OH)_2D_3$ can be regarded as a hormone derived from vitamin D, whose synthesis is feedback regulated in a complex manner involving serum calcium, phosphorus, and parathyroid hormone. Evidence in experimental animals suggests that patients suffering from chronic renal disease, vitamin D-dependency disease, and hypoparathyroidism have a failure in the 1-hydroxylating mechanism.

The findings of vitamin D metabolism in experimental animals has led to the initiation of a search for similar systems in man. In addition, metabolites of vitamin D and their analogs are now being studied for potential use in the treatment of vitamin D-resistant bone disease. It seems likely that basic investigation into the metabolism and function of vitamin D will lead to new understanding of metabolic bone disease and to practical new methods of management in the clinics.

ACKNOWLEDGMENT

Some of the original investigations described in this review and its preparation were supported by grants from the National Institutes of Health Nos. AM-14881 and AM-15512, and a contract from the U.S. Atomic Energy Commission No. AT-(11-1)1668.

Literature Cited

1. Mellanby, E. 1919. *Lancet* 1:407–12
2. McCollum, E. V., Simmonds, N., Becker, J. E., Shipley, P. G. 1922. *J. Biol. Chem.* 53:293–312
3. Huldshinsky, K. 1919. *Deut. Med. Wochenschr.* 45:712–13
4. Hess, A. F., Weinstock, M., Helman, F. D. 1925. *J. Biol. Chem.* 63:305–8
5. Steenbock, H., Black, A. 1924. *J. Biol. Chem.* 61:405–22
6. Steenbock, H. 1924. *Science* 60:224–25
7. Howland, J., Kramer, B. 1921. *Am. J. Dis. Child.* 22:105–19
8. Neuman, W. F. 1958. *Am. Med. Assoc. Arch. Pathol.* 66:204–9
9. Nicolaysen, R. 1937. *Biochem. J.* 31:122–29
10. Nicolaysen, R. 1943. *Acta Physiol. Scand.* 6:201–9
11. Harrison, H. C., Harrison, H. E. 1969. *Am. J. Physiol.* 217:121–25
12. Wensel, R. H. et al 1969. *J. Clin. Invest.* 48:1768–75
13. Wasserman, R. H. 1963. *The Transfer of Calcium and Strontium Across Biological Membranes*, ed. R. H. Wasserman, 211–28. New York: Academic

14. Wasserman, R. H. 1970. *The Fat-Soluble Vitamins,* ed. H. F. DeLuca, J. W. Suttie, 21–37. Madison: Univ. Wisc.
15. Wasserman, R. H., Taylor, A. N. 1972. *Ann. Rev. Biochem.* 41:179–202
16. Omdahl, J. L., DeLuca, H. F. 1973. *Physiol. Rev.* 53:327–72
17. Carlsson, A. 1952. *Acta Physiol. Scand.* 26:212–20
18. Harrison, H. C., Harrison, H. E., Park, E. A. 1958. *Am. J. Physiol.* 192:432–36
19. Rasmussen, H., DeLuca, H., Arnaud, C., Hawker, C., von Stedingk, M. 1963. *J. Clin. Invest.* 42:1940–46
20. Harrison, H. E., Harrison, H. C. 1941. *J. Clin. Invest.* 20:47–55
21. Puschett, J. B., Moranz, J., Kurnick, W. S. 1972. *J. Clin. Invest.* 51:373–85
22. Puschett, J. B. et al 1972. *Proc. Soc. Exp. Biol. Med.* 141:379–84
23. Brodehl, J., Kaas, W. P., Weber, H.-P. 1971. *Pediat. Res.* 5:591–604
24. Cruickshank, E. M., Kodicek, E. 1953. *Biochem. J.* 54:337–43
25. Kodicek, E. 1955. *Biochem. J.* 60:XXV
26. Kodicek, E. 1956. *Ciba Foundation Symposium on Bone Structure and Metabolism,* ed. G. W. E. Wolstenholme, C. M. O'Connor, 161–74. Boston:Little Brown
27. Norman, A. W., DeLuca, H. F. 1963. *Biochemistry* 2:1160–68
28. Neville, P. F., DeLuca, H. F. 1966. *Biochemistry* 5:2201–7
29. Lund, J., DeLuca, H. F. 1966. *J. Lipid Res.* 7:739–44
30. Morii, H., Lund, J., Neville, P. F., DeLuca, H. F. 1967. *Arch. Biochem. Biophys.* 120:508–12
31. Blunt, J. W., DeLuca, H. F., Schnoes, H. K. 1968. *Biochemistry* 7:3317–22
32. Holick, M. F., DeLuca, H. F., Avioli, L. V. 1972. *Arch. Intern. Med.* 129:56–61
33. Ponchon, G., DeLuca H. F. 1969. *J. Clin. Invest.* 48:1273–79
34. Ponchon, G., Kennan, A. L., DeLuca, H. F. 1969. *J. Clin. Invest.* 48:2032–37
35. Horsting, M., DeLuca, H. F. 1969. *Biochem. Biophys. Res. Commun.* 36:251–56
36. DeLuca, H. F. 1969. *Am. J. Clin. Nutr.* 22:412–24
37. Suda, T. et al 1970. *Biochemistry* 9:2917–22
38. Suda, T., DeLuca, H. F., Schnoes, H. K., Tanaka, Y., Holick, M. F. 1970. *Biochemistry* 9:4776–80
39. Holick, M. F. et al 1972. *Biochemistry* 11:4251–55
40. Haussler, M. R., Myrtle, J. F., Norman, A. W. 1968. *J. Biol. Chem.* 243:4055–64
41. Ponchon, G., DeLuca, H. F. 1969. *J. Nutr.* 99:157–67
42. Lawson, D. E. M., Wilson, P. W., Kodicek, E. 1969. *Biochem. J.* 115:269–77
43. Cousins, R. J., DeLuca, H. F., Suda, T., Chen, T., Tanaka, Y. 1970. *Biochemistry* 9:1453–59
44. Cousins, R. J., DeLuca, H. F., Gray, R. W. 1970. *Biochemistry* 9:3649–52
45. Haussler, M. R., Boyce, D. W., Littledike, E. T., Rasmussen, H. 1971. *Proc. Nat. Acad. Sci. USA* 68:177–81
46. Omdahl, J., Holick, M., Suda, T., Tanaka, Y., DeLuca, H. F. 1971. *Biochemistry* 10:2935–40
47. Myrtle, J. F., Norman, A. W. 1971. *Science* 171:79–82
48. Kodicek, E., Lawson, D. E. M., Wilson, P. W. 1970. *Nature* 228:763–64
49. Holick, M. F., Schnoes, H. K., DeLuca, H. F. 1971. *Proc. Nat. Acad. Sci. USA* 68:803–4
50. Holick, M. F., Schnoes, H. K., DeLuca, H. F., Suda, T., Cousins, R. J. 1971. *Biochemistry* 10:2799–804
51. Lawson, D. E. M., Fraser, D. R., Kodicek, E., Morris, H. R., Williams, D. H. 1971. *Nature* 230:228–30
52. Fraser, D. R., Kodicek, E. 1970. *Nature* 228:764–66
53. Gray, R., Boyle, I., DeLuca, H. F. 1971. *Science* 172:1232–34
54. Norman, A. W., Midgett, R. J., Myrtle, J. F., Nowicki, H. G. 1971. *Biochem. Biophys. Res. Commun.* 42:1082–87
55. Boyle, I. T., Miravet, L., Gray, R. W., Holick, M. F., DeLuca, H. F. 1972. *Endocrinology* 90:605–8
56. Holick, M. F., Garabedian, M., DeLuca, H. F. 1972. *Science* 176:1146–47
57. Wong, R. G., Norman, A. W., Reddy, C. R., Coburn, J. W. 1972. *J. Clin. Invest.* 51:1287–91
58. Bhattacharyya, M. H., DeLuca, H. F. 1973. *J. Biol. Chem.* 248:2969–73
59. Gray, R. W., Omdahl, J. L., Ghazarian, J. G., DeLuca, H. F. 1972. *J. Biol. Chem.* 247:7528–32
60. Ghazarian, J. G., Schnoes, H. K., DeLuca, H. F. 1973. *Biochemistry* 12:2555–58
61. Omdahl, J. L., Gray, R. W., Boyle, I. T., Knutson, J., DeLuca, H. F. 1972. *Nature New Biol.* 237:63–64
62. Avioli, L. V., Lee, S. W., McDonald, J. E., Lund, J., DeLuca, H. F. 1967. *J. Clin. Invest.* 46:983–92

63. Avioli, L. V., Birge, S., Lee, S. W., Slatopolsky, E. 1968. *J. Clin. Invest.* 47:2239–52
64. Stanbury, S. W. et al 1972. *Endocrinology 1971,* ed. S. Taylor, 487–99. London:Heinemann
65. Mawer; E. B., Backhouse, J., Holman, C. A., Lumb, G. A., Stanbury, S. W. 1972. *Clin. Sci.* 43:413–31
66. Rosenstreich, S. J., Rich, C., Volwiler, W. 1971. *J. Clin. Invest.* 50:679–87
67. Higaki, M., Takahashi, M., Suzuki, T., Sahashi, Y. 1965. *J. Vitaminol.* 11:261–65
68. Mawer, E. B., Backhouse, J., Lumb, G. A., Stanbury, S. W. 1971. *Nature New Biol.* 232:188–89
69. Schaefer, K., von Herrath, D., Stratz, R. 1972. *Israel J. Med. Sci.* 8:80–83
70. Belsey, R., DeLuca, H. F., Potts, J. T. Jr. 1971. *J. Clin. Endocrinol. Metab.* 33:554–57
71. Belsey, R., DeLuca, H. F., Potts, J. T. Jr. 1972. *Calcium, Parathyroid Hormone and the Calcitonins,* ed. R. V. Talmage, P. L. Munson, 236–39. Amsterdam:Excerpta Medica
72. Haddad, J. G., Chyu, K. J. 1971. *J. Clin. Endocrinol.* 33:992–95
73. Stamp, T. C. B., Round, J. M., Rowe, D. J. F., Haddad, J. G. 1973. *Brit. Med. J.* 4:9–12
74. Haddad, J. G. Jr., Chyu, K. J., Hahn, T. J., Stamp. T. C. B. 1973. *J. Lab. Clin. Med.* 81:22–27
75. Chalk, K. J. I., Kodicek, E. 1961. *Biochem. J.* 79:1–4
76. Rikkers, H., DeLuca, H. F. 1967. *Am. J. Physiol.* 213:380–86
77. Crousaz, P. D., Blanc, B., Antener, I. 1965. *Helv. Odontol. Acta* 9:151–55
78. Peterson, P. A. 1971. *J. Biol. Chem.* 246:7748–54
79. Frolik, C. A., DeLuca, H. F. 1972. *J. Clin. Invest.* 51:2900–6
80. Frolik, C. A., DeLuca, H. F. 1971. *Arch. Biochem. Biophys.* 147:143–47
81. Wong, R. G., Myrtle, J. F., Tsai, H. C., Norman, A. W. 1972. *J. Biol. Chem.* 247:5728–35
82. Boyle, I. T., Omdahl, J. L., Gray, R. W., DeLuca, H. F. 1973. *J. Biol. Chem.* 248:4174–80
83. Boyle, I. T., Gray, R. W., DeLuca, H. F. 1971. *Proc. Nat. Acad. Sci. USA* 68:2131–34
84. Holick, M. F., Kleiner-Bossaller, A., Schnoes, H. K., Kasten, P. M., DeLuca, H. F. 1973. *J. Biol. Chem.* 248: 6691–96
85. Bhattacharyya, M. H., DeLuca, H. F. 1973. *J. Biol. Chem.* 248:2974–77
86. Trummel, C. L., Raisz, L. G., Blunt, J. W., DeLuca, H. F. 1969. *Science* 163:1450–51
87. Pavlovitch, H., Garabedian, M., Balsan, S. 1973. *J. Clin. Invest.* 52: 2656–60
88. Nicolaysen, R., Eeg-Larsen, N., Malm, O. J. 1953. *Physiol. Rev.* 33:424–44
89. Rottensten, K. V. 1938. *Biochem. J.* 32:1285–92
90. Fairbanks, B. W., Mitchell, H. H. 1936. *J. Nutr.* 11:551–72
91. Boyle, I. T., Gray, R. W., Omdahl, J. L., DeLuca, H. F. 1972. *Endocrinology 1971,* ed. S. Taylor, 468–76. London: Heinemann
92. Corradino, R. A., Wasserman, R. H. 1970. *Proc. Soc. Exp. Biol. Med.* 133:960–63
93. Omdahl, J. L., DeLuca, H. F. 1971. *Science* 174:949–51
94. Omdahl, J. L., DeLuca, H. F. 1972. *J. Biol. Chem.* 247:5520–26
95. Garabedian, M., Holick, M. F., DeLuca, H. F., Boyle, I. T. 1972. *Proc. Nat. Acad. Sci. USA* 69:1673–76
96. Rasmussen, H., Wong, M., Bikle, D., Goodman, D. B. P. 1972. *J. Clin. Invest.* 51:2502–4
97. Fraser, D. R., Kodicek, E. 1973. *Nature New Biol.* 241:163–66
98. Hill, L. F., Mawer, E. B. 1972. *Abstr. Med. Res. Soc. Meeting, London*
99. Galante, L., Colston, K. W., MacAuley, S. J., MacIntyre, I. 1972. *Nature* 238:271–73
100. Galante, L., MacAuley, S. J., Colston, K. W., MacIntyre, I. 1972. *Lancet* i:985–88
101. Tanaka, Y., DeLuca, H. F. 1973. *Arch. Biochem. Biophys.* 154:566–74
102. Morrissey, R. L., Wasserman, R. H. 1971. *Am. J. Physiol.* 220:1509–15
103. Tanaka, Y., Frank, H., DeLuca, H. F. 1973. *Science* 181:564–66
104. Tanaka, Y., Chen, T. C., DeLuca, H. F. 1972. *Arch. Biochem. Biophys.* 152:291–98
105. Kruse, R. 1968. *Monatsschr. Kinderheilk.* 116:378–81
106. Richens, A., Rowe, D. J. F. 1970. *Brit. Med. J.* 4:73
107. Dent, C. E., Richens, A., Rowe, D. J. F., Stamp. T. C. B. 1970. *Brit. Med. J.* 4:69–72
108. Hahn, J. J., Birge, S. J., Scharp, C. R., Avioli, L. V. 1972. *J. Clin. Invest.* 51:741–48

109. Conney, A. H. 1967. *Pharmacol. Rev.* 19:317
110. Hunter, J., Maxwell, J. D., Stewart, D. A., Parsons, V., Williams, R. 1971. *Brit. Med. J.* 4:202–4
111. Hahn, T. J., Hendin, B. A., Scharp, G. R., Haddad, J. G. 1972. *N. Engl. J. Med.* 287:900–4
112. Hahn, T. J. 1973. *Ann. Intern. Med.* 78:308–9
113. Harrison, H. E., Harrison, H. C. 1960. *Am. J. Physiol.* 199:265–71
114. Williams, G. A., Bowser, E. N., Henderson, W. J., Uzgiries, V. 1961. *Proc. Soc. Exp. Biol. Med.* 106:664–66
115. Sallis, J. D., Holdsworth, E. S. 1962. *Am. J. Physiol.* 203:506–12
116. Anderson, J., Dent, C. E., Harper, C., Philpot, G. R. 1954. *Lancet* 2:720–24
117. Bell, N. H., Gill, J. R., Bartter, F. C. 1964. *Am. J. Med.* 36:500–13
118. Verner, J. V., Englel, F. L., McPherson, H. T. 1958. *Ann. Intern. Med.* 48:765–73
119. Avioli, L. V., Birge, S. J., Lee, S. W. 1968. *J. Clin. Endocrinol.* 28:1341–46
120. Kimberg, D. V., Baerg, R. D., Gershon, E., Grandusius, R. T. 1971. *J. Clin. Invest.* 50:1309–21
121. Blunt, J. W., Tanaka, Y., DeLuca, H. F. 1968. *Proc. Nat. Acad. Sci. USA* 61:1503–6
122. Balsan, S., Garabedian, M. 1972. *J. Clin. Invest.* 51:749–59
123. Earp, H. S., Ney, R. L., Gitelman, H. J., Richman, R., DeLuca, H. F. 1970. *N. Engl. J. Med.* 283:627–30
124. Pak, C. Y. C. et al 1972. *Arch. Intern. Med.* 129:894–99
125. Brickman, A. S., Coburn, J. W., Norman, A. W. 1972. *N. Engl. J. Med.* 287:891–95
126. Fraser, D. et al 1973. *N. Engl. J. Med.* 289:817–22
127. Glorieux, F. H., Scriver, C. R., Holick, M. F., DeLuca, H. F. 1973. *Lancet.* II:287–89
128. Roborgh, J. R., DeMan, T. J. 1960. *Biochem. Pharmacol.* 3:277–82
129. Suda, T., Hallick, R. B., DeLuca, H. F., Schnoes, H. K. 1970. *Biochemistry* 9:1651–57
130. Hallick, R. B., DeLuca, H. F. 1972. *J. Biol. Chem.* 247:91–97
131. Harrison, H. E., Harrison, H. C. 1972. *J. Clin. Invest.* 51:1919–22
132. Holick, M. F., DeLuca, H. F., Kasten, P. M., Korycka, M. B. 1973. *Science* 180:964–66
133. Holick, M. F., Garabedian, M., DeLuca, H. F. 1972. *Science* 176:1247–48
134. Holick, M. F., Garabedian, M., DeLuca, H. F. 1972. *Biochemistry* 11:2715–19
135. Steele, T. H. et al 1973. *Abstr. Proc. Am. Soc. Clin. Invest.*, p. 81a
136. Rutherford, E. et al 1973. *Abstr. Clin. Res.* In press
137. Semmler, E. J., Holick, M. F., Schnoes, H. K., DeLuca, H. F. 1972. *Tetrahedron Lett.* 40:4147–50
138. Holick, M. F., Semmler, E. J., Schnoes, H. K., DeLuca, H. F. 1973. *Science* 180:190–91

Copyright 1974. All rights reserved

STATIC (ISOMETRIC) EXERCISE AND THE HEART: Physiological and Clinical Considerations[1]

❖7119

Jere H. Mitchell, M.D. and Kern Wildenthal, M.D., Ph.D.
Pauline and Adolph Weinberger Laboratory for Cardiopulmonary Research, Departments of Internal Medicine and Physiology, The University of Texas Southwestern Medical School, Dallas, Texas

INTRODUCTION

During the performance of muscular exercise, the heart and the vascular system undergo important adaptive changes. The exact nature of these changes depends to a large extent on the specific type of exertion undertaken. Broadly speaking, there are two types of muscular activity. Exercise in which skeletal muscle contraction causes principally a change in length with little change in tension is termed dynamic or isotonic, and that in which the contraction causes principally a change in tension with little change in length is termed static or isometric. Most muscular exercise is neither purely dynamic nor purely static. Activities that are predominantly dynamic include running, swimming, bicycling, rowing, and rhythmic calisthenics, and those predominantly static include lifting or pushing heavy weights and contracting muscles against fixed objects.

During dynamic exercise involving a large mass of skeletal muscle, there is great demand for oxygen to supply the increased metabolic needs of the contracting muscles. This type of activity results in large increases in cardiac output, heart rate, and stroke volume, with relatively little change in mean arterial pressure. Systolic pressure tends to increase but diastolic pressure does not. Static contraction, on the other hand, of even a small mass of muscle causes a marked increase in mean arterial pressure with relatively small increases in heart rate and cardiac output. Both systolic and diastolic pressures are increased. Thus, dynamic exercise may be thought of as causing primarily a volume load on the heart, whereas static exercise produces primarily a pressure load.

---

[1] Supported by the Harry S. Moss Heart Fund and NASA grant NGR 44-012-151.

Historically, there has been extensive interest in and investigation of the cardiac effects of exercise in health and disease, but the form of exercise that has been studied almost exclusively has been dynamic exercise. Only in the last decade has the growing appreciation of the special characteristics and importance of isometric exercise stimulated a number of systematic investigations into the subject, and only very recently have general reviews of the topic been available (1, 2). The purpose of the present review is to describe what is known of the effects of static exercise on the heart, with special emphasis on the physiological control mechanisms involved in the cardiovascular responses. In addition, implications for the clinical use of isometric exercise in evaluating cardiac function and the special consequences of this type of muscular activity in patients with cardiovascular disease are considered.

CARDIOVASCULAR EFFECTS OF STATIC EXERCISE

During the first half of this century, occasional studies were made of partially static exercise, but only a few dealt systematically with the topic. It was generally assumed that the most important special aspect of static exertion was that blood flow to the muscle was inadequate due to mechanical occlusion of the artery by the contracting fibers. In 1920 Lindhard (3) studied in some detail the cardiovascular adjustments that occur when human volunteers performed heavy and prolonged weight-bearing exertion. He observed an increase in oxygen uptake and cardiac output, particularly after the cessation of exertion; the changes were less than would be expected to occur during dynamic exercise that resulted in a similar degree of exhaustion. Later, in 1937 and 1938, Alam & Smirk (4, 5) published the results of an important series of experiments in which human subjects performed predominantly isometric exercise. They found that mild tachycardia and dramatic increases in arterial pressure invariably accompanied the exertion. Also, in 1938 Asmussen & Hansen (6) showed that isometric exercise in the leg produced increases in oxygen uptake, cardiac output, heart rate, and blood pressure. They, as well as Alam & Smirk, observed that cessation of the exercise was normally accompanied by a rapid return to normal heart rate and blood pressure even though cardiac output and oxygen uptake might remain elevated. The return of heart rate and blood pressure toward control values could be delayed by circulatory occlusion to the exercising limb (4, 5). Subsequently, Tuttle & Horvath (7) confirmed many of these earlier observations and emphasized the differences between the effects of static and dynamic exertion, particularly with regard to the diastolic blood pressure.

It was not until the 1960s, however, that an extensive and comprehensive investigation of the special characteristics of isometric effects was undertaken. The elegant series of papers that appeared from the laboratories of Lind, Donald, and colleagues (8–17), along with that of Freyschuss (18), remain the primary sources of basic information on the physiology of cardiovascular responses to isometric exercise. In those studies graded degrees of handgrip contraction were used to show conclusively not only that an unexpectedly high rise in blood pressure occurs, but also that the extent of the rise is a predictable function of the intensity of the isometric effort, expressed as a percentage of the maximal voluntary contraction. Similarly, increases in heart rate and cardiac output are closely related to the degree of exertion. It is of special interest that neither the absolute tension development nor the size of the muscle group is the determining factor; rather, the important element seems to be the percentage of maximal contraction by the particular muscle group involved. Thus, blood pressure rises to a similar extent with 20% of maximal handgrip, 20% of maximal contraction by thigh muscles, or 20% of maximal adduction of individual fingers, despite the manyfold differences in absolute tension developed and in the energy requirements of the respective efforts (13, 14).

A practical consequence of this observation is that a given load can be carried by a large, strong muscle group (e.g. those of the shoulder) without causing as much cardiovascular stress as when the same absolute load is carried by a smaller, weaker group of muscles, such as the hand (16, 17). It is also of note in this regard that contractions by multiple muscle groups are not additive in their effects. When muscles of both hands (or of a hand and a leg, etc) contract to the same degree, the net cardiovascular effect is identical to that observed with a contraction by one hand alone (13). Thus, if a 40-lb weight is carried as a single load in one hand, the resultant changes in blood pressure, heart rate, etc are much greater than if the weight is distributed as two 20-lb loads carried in both hands. The latter effort yields identical changes to those resulting from carrying a single 20-lb weight in one hand (16, 17).

In addition to the degree of effort involved, the duration of isometric contraction is an important determinant of the cardiovascular changes that ensue (9). With contractions of less than 15% of maximal voluntary effort, a new steady state may develop after several minutes. With stronger contractions, however, the blood pressure, heart rate, and cardiac output invariably continue to rise until muscular fatigue prohibits continued contraction. In keeping with earlier suppositions, it is now clear that stronger contractions can cause mechanical interference to blood flow to the muscle (8, 19, 20), and the resultant ischemia presumably contributes to the rapid development of fatigue. In general, the rate at which hemodynamic factors change during exertion and the final extent of their change are directly related to the strength of muscle contraction and inversely related to the time required to produce fatigue. The intensity of exertion also correlates well with the degree of ATP, creatine phosphate, and glycogen depletion, and with the accumulation of lactate and other glycolytic intermediates (21, 22).

During the past five years evaluation of isometric exercise responses has been performed in many laboratories during left heart catheterization, and the normal response to varying levels of exertion has been defined (23–29). As a rule, the marked increase in afterload that results from the exertion is accompanied by an increase in left ventricular contractile force, however measured. There is no change or only a small rise (< 5 mm Hg) in left ventricular end-diastolic pressure, implying that the Starling mechanism is not required in a major way to meet the imposed stress. The extent of rise of end-diastolic pressure varies with the pressure load, as would be expected. Mullins & Blomqvist (28) have described typical responses in a patient with relatively normal left ventricular function and these are shown in Figure 1. At both 25% and 50% of maximal voluntary contraction in a patient with a calcified mitral annulus, there was a marked increase in left ventricular systolic pressure and a moderate increase in heart rate. Stroke work index increased significantly while left ventricular end-diastolic pressure remained constant at 25% of maximal voluntary contraction and only increased slightly at 50%.

The strength and dominance of the isometric responses are impressive. They persist, undiminished, during dynamic exercise (13, 30). Similarly, induction of the diving reflex, which reverses the tachycardia of dynamic exercise, does not inhibit the ability of isometric exercise to raise the heart rate (31). Also, elicitation of the isometric response serves to overwhelm the baroreceptor reflex, so that even that usually dominating homeostatic mechanism in markedly inhibited (32).

The individual cardiovascular factors that contribute to the net blood pressure response are not always identical in all subjects. In any given individual, the rise in arterial pressure can be the result of increased cardiac output, increased peripheral resistance, or both, though in most subjects the cardiac output response seems to predominate (1, 14). The increased cardiac output usually results primarily from

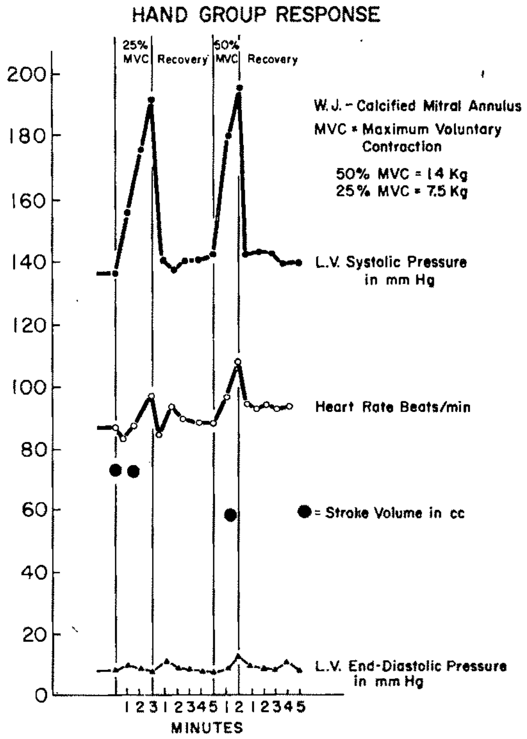


Figure 1 Hemodynamic responses in a patient with normal left ventricular function during 25% and 50% of maximal handgrip contraction. [Reprinted from Mullins & Blomqvist (28), with the permission of the publishers.]

tachycardia but may also be caused in part by an increased stroke volume in some individuals. The efferent mechanism that mediates the heart rate changes remains controversial (1, 11, 14, 18). On the basis of pharmacologic blocking experiments, Macdonald et al (11) concluded that sympathetic stimulation is the major factor, whereas Freyschuss (18) believed that vagal withdrawal is a more important determinant. If, for various pharmacological or pathological reasons, one or more of the above changes is prevented from developing, another often seems to fill the breach. Thus, as Donald et al (14) have observed, when isometric exercise calls forth an elevation of blood pressure, "it would appear that the higher cardiovascular centers will, if necessary, recruit all available mechanisms to achieve this."

CARDIOVASCULAR CONTROL MECHANISMS DURING STATIC EXERCISE

The mechanisms that serve to initiate the increases in blood pressure, heart rate, and cardiac output during static exercise have not been defined completely. The rapid onset of the cardiac changes strongly suggests a neurogenic mechanism (18, 33–35). Over the years, two general hypotheses of neurogenic control of cardiovascular responses to exercise have evolved. One of these holds that the responses are due to a direct action of the motor cortex on the cardiovascular center and the other that the responses are due to a reflex that originates in the exercising skeletal muscle.

The hypothesis that the cardiovascular responses during exercise are due to a direct action of the motor cortex on the cardiovascular center (cortical irradiation) was elaborated by Krogh & Lindhard in 1913 (36). Asmussen, Christensen & Nielsen (37) thought they had found support for this hypothesis when they showed that the cardiac output response during dynamic leg exercise was related to the intensity of the work, even when circulation to the legs was blocked and the oxygen consumption was halved. Later, Asmussen, Nielsen & Wieth-Pedersen (38) compared the cardiovascular responses to leg exercise performed voluntarily with that produced by direct electrical stimulation of the muscles. They showed that blood pressure, heart rate, and cardiac output response was increased in relation to the oxygen consumption independent of how the exercise was produced. From these later studies they concluded that cortical irradiation played no role in the regulation of circulation during steady state muscular exercise.

Ochwadt et al (39) and Asmussen et al (40) found that heart rate and blood pressure responses were greater at a given work load when muscular strength was reduced by partial curarization. Thus the cardiovascular response was related to the greater motor command needed to achieve a given level of muscular work when the subject was weaker. This finding suggested that cortical irradiation was involved, even though Asmussen et al (40) favored an alternative explanation. They suggested that a greater activity in the afferent nerves from muscle spindles was responsible for the enhanced cardiovascular responses. However, McCloskey, Matthews & Mitchell (41) demonstrated in anesthetized and decerebrated cats that activation of muscle spindle primary afferents by vibration causes no change in blood pressure or heart rate. That afferents from muscle spindles are not involved in the response during isometric exercise was also demonstrated by the nerve blocking studies of McCloskey & Mitchell (42) and the afferent recording studies of Pérez-González & Coote (43).

Freyschuss demonstrated an increase in blood pressure and heart rate in subjects whose muscles were unable to respond to command signals to perform handgrip (18). The muscular groups involved in handgrip were paralyzed by injection of succinylcholine into the brachial artery. The cardiovascular responses to intended isometric handgrip were less than appropriate control data.

Goodwin, McCloskey & Mitchell investigated the cardiovascular response of human subjects to isometric exercise in experiments where the central motor command required to achieve a given tension was varied (44). High-frequency vibration of a muscle excites the primary afferents of muscle spindles. If the spindle primary afferents are activated in a contracting muscle, they reflexly cause an involuntary increase in motor activation of the muscle, so that less central command is required to maintain the same tension. On the other hand, if the spindle afferents are activated in the antagonists of a contracting muscle, they cause reflex inhibition of the contracting muscle, so that a greater central command is required and the subject perceives that the conscious effort has increased. When the same muscle tension was achieved with less central command during isometric exercise the resultant hypertension and tachycardia decreased. The responses of blood pressure and heart rate for two levels of muscular tension during isometric exercise with greater central command are shown in Figure 2. During this study blood pressure and heart rate increases were greater when the subjects perceived that greater effort was required, even though the true level of developed tension was identical. It was concluded, therefore, that there is radiation of cardiovascular control centers by the descending central command during voluntary isometric contraction in man.

The hypothesis that the cardiovascular responses during exercise are due to a reflex originating in contracting skeletal muscle received its first major experimental support in the work of Alam & Smirk (4, 5). They demonstrated an increase in blood pressure and in heart rate while the leg was performing predominantly isometric exercise during local circulatory occlusion. The responses remained after exercise stopped, until the vascular occlusion was removed. They concluded that receptors located in the skeletal muscles were activated by some byproduct of the contraction and stimulated a reflex increase in blood pressure and heart rate. Similar conclusions were reached by Staunton, Taylor & Donald (10). Further evidence that a reflex mechanism was responsible for the cardiovascular changes during isometric exercise was found in the study of a patient with syringomyelia predominantly involving one of the upper extremities (15). Static exercise of the uninvolved forearm caused an increase in blood pressure and heart rate but activity of the affected forearm caused no increase in blood pressure and a smaller increase in heart rate.

Studies in experimental animals have also demonstrated a cardiovascular reflex originating in skeletal muscle. Kao has shown that the artificial production of rhythmic exercise in the hind limbs of dogs caused cardiovascular responses that were partly due to stimulation of receptors located in the exercising muscle (45, 46). Coote et al (47) caused isometric exercise in cats by stimulating spinal ventral roots

L_6-S_1 before and after sectioning the corresponding dorsal roots. Before cutting the dorsal roots, exercise caused an increase in blood pressure and heart rate, whereas no response was apparent after the dorsal (sensory) roots were cut. This demonstrated that the response was reflex in nature with the afferent limb located in the dorsal root.

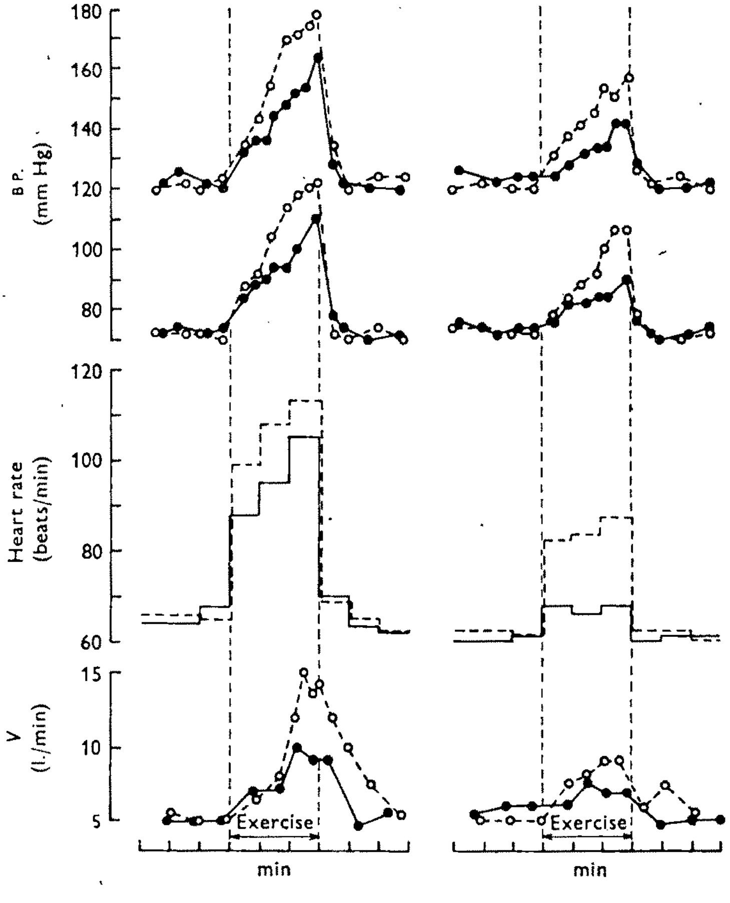


Figure 2 Cardiovascular responses during isometric exercise at a constant muscle tension with an increased central command. Systolic and diastolic blood pressures (BP), heart rate, and minute ventilation are plotted before, during, and after two isometric contractions of the triceps at 35% (*left panel*) and 15% (*right panel*) of maximal voluntary contraction. Responses are shown to normal contractions (●——●) and when the central command to triceps was increased by inhibition of biceps (o - - o). [Reprinted from Goodwin, McCloskey & Mitchell (44), with the permission of the publishers.]

During induced isometric exercise McCloskey & Mitchell (42) observed similar cardiovascular responses which were abolished by dorsal root section, and they proceeded to determine the type of afferent nerve fiber that carried the reflex impulses. Preferential blockade of large myelinated fibers by the direct current anodal block technique did not abolish the evoked cardiovascular responses; on the other hand, blockade of small myelinated and unmyelinated fibers by local anesthetic application abolished the responses completely. It was concluded that the reflexly induced cardiovascular responses during muscular contraction are mediated by fibers within groups III and IV (small myelinated and unmyelinated fibers). Other experiments have demonstrated that electrical activation of afferent fibers from muscle can elicit cardiac responses similar to those that accompany exercise, and these studies also suggest that the fibers involved are probably unmyelinated or small myelinated ones (48–51). The changes even include an increase in left ventricular contractile state (50).

The mechanism by which the receptor attached to type III or type IV fibers is activated during muscular contraction is not known. Since the original studies of Alam & Smirk (4, 5), strong circumstantial evidence has been accumulated suggesting that chemical factors may play a major role, presumably as a result of accumulation of local metabolites. The specific chemical or chemicals that may be involved remain undefined. Wildenthal and co-workers (52) reported a significant increase in arterial pressure, heart rate, cardiac output, and left ventricular contractility during infusion of small amounts of potassium ion into the vascularly isolated, innervated dog hind leg and suggested that increases in extracellular [K^+], known to occur during exercise (53–55), may initiate cardiotonic reflexes from exercising muscles. This suggestion was in agreement with the prior observation of Donald et al (14) that K^+ release from muscle into the venous blood was the only metabolic event they had observed that correlated well, temporally and quantitatively, with the cardiovascular responses to isometric exercise. Hník et al (56) have documented that the afferent fibers activated by K^+ are indeed small group III and group IV fibers similar to those activated by muscular exercise.

Wildenthal et al (57) have also suggested that, in addition to a specific elevation of [K^+] in the muscle tissue, the nonspecific increase in local osmolality that accompanies exercise (58) may help elicit cardiotonic and pressor reflexes, since local infusion of hypertonic solutions in experimental animals is appropriately reflexogenic (52, 59). Stegemann and co-workers (60) reported reflex tachycardia and hypertension after CO_2 was bubbled into the abdominal aorta of dogs, and they suggested that local hypercarbia might contribute to the exercise-induced reflexes.

In summary, whatever the specific reflexogenic agent or combination of agents may prove to be, current evidence suggests that part of the increase in heart rate, blood pressure, and cardiac contractility that accompanies static muscle contraction is mediated by reflexes arising in the exercising muscle itself. In addition, there is sound evidence that central command also plays an important role, so available data suggest that both cortical irradiation and reflex arcs contribute to the neurogenic control of cardiovascular changes during static exercise. The relative contributions of each of these two mechanisms are not known.

DIAGNOSTIC USE OF STATIC EXERCISE IN CARDIOVASCULAR DISEASE

Brief periods of isometric exercise are now recognized to be useful in clinical evaluations of left ventricular performance. Both noninvasive techniques (61–64) and left heart catheterization (23–29) have been used to test the ability of the left ventricle to adapt to the pressure stress that accompanies static muscular contraction.

Phonocardiography has been suggested to be useful during isometric exercise for eliciting or augmenting third and fourth heart sounds in patients with arteriosclerotic heart disease (62, 63). Sustained handgrip has also been reported useful in altering the murmurs of aortic stenosis and distinguishing valvular from subaortic obstruction (64). Use of systolic time intervals, on the other hand, is not particularly helpful diagnostically, inasmuch as changes during isometric exercise are similar in normals and patients with heart disease (61, 62).

Isometric exercise appears to have potential value particularly as a cardiac stress test. In certain respects it may be superior to angiotensin infusions in this regard (65). Several groups have now reported that ventricular function curves drawn from values obtained at rest and during handgrip are steep in normal subjects and tend to be less steep or flat in patients with heart disease (23–29). In patients with good cardiac function there is a steep rise in stroke work with little change in left ventricular end-diastolic pressure, indicating an increase in the contractile state. In patients with poor cardiac function there is a marked rise of left ventricular end-diastolic pressure with little or no increase in stroke work, showing that the left ventricle has little or no physiological capacity to improve its contractile state and is operating on a depressed Frank-Starling curve. Results are similar when ventricular performance is evaluated by simple function curves or by derived indices which supposedly measure contractile element behavior (66).

Changes that occur in heart rate during isometric exercise may make evaluation of ventricular function in cardiac patients difficult at times. Therefore, Amende et al (27) kept heart rate constant by electrical pacing during the isometric handgrip test. The results of their study are shown in Figure 3. Six patients (group I) had a normal left ventricular end-diastolic pressure at rest and responded to handgrip with a significant increase in left ventricular stroke work index with an essentially unchanged end-diastolic pressure; they were considered to be normal. Ten patients (group III) had an increased left ventricular end-diastolic pressure at rest and responded to handgrip with a significant increase in left ventricular end-diastolic pressure with an insignificant increase in stroke work index; they were obviously abnormal. Nine patients (group II) had a normal left ventricular end-diastolic pressure at rest but demonstrated a significant increase in both end-diastolic pressure and stroke work index during handgrip; this group would appear to have abnormal left ventricular function. It is in this identification of patients that may seem to have normal ventricular function at rest, but who develop abnormalities when stressed, that isometric exercise probably has an especially useful clinical role. Jackson (67) has suggested that it may have a similarly useful role even without sophisticated hemodynamic monitoring in angina patients. He thought that the pressure load imposed on the heart might serve as a useful stress test, just as dynamic exercise does; however, others have not found isometric exertion to be as useful as dynamic exercise in angina patients (62).

STATIC EXERCISE IN PATIENTS WITH CARDIOVASCULAR DISEASE

In recent years there has been much popular interest in isometric exertion as a quick and easy way for busy city-dwellers to exercise (68). However, even intense and prolonged programs of isometric training lead to little or no significant increase in maximal oxygen uptake or cardiac reserve (69). Thus, it should be emphasized that,

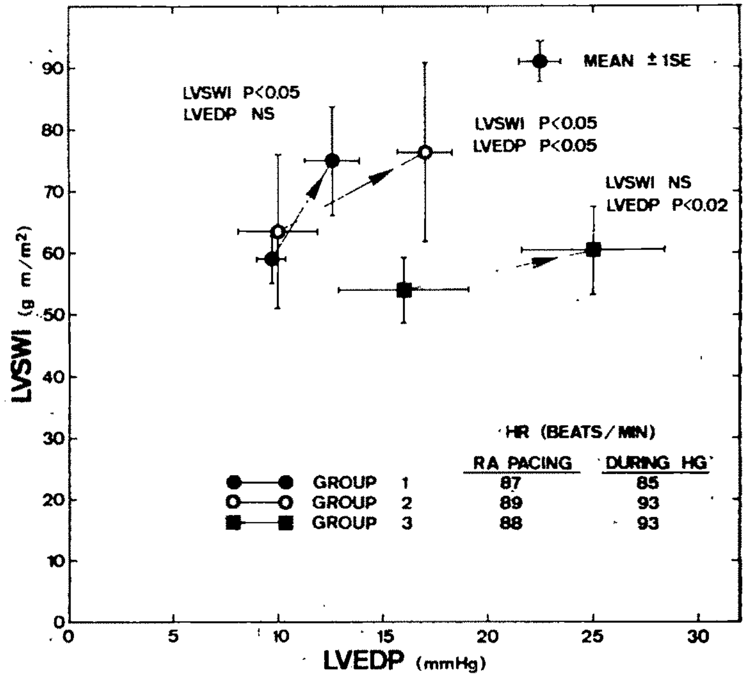


Figure 3 Relation between left ventricular stroke work index and end-diastolic pressure during right atrial pacing and during handgrip. LVSWI = left ventricular stroke work index. LVEDP = left ventricular end-diastolic pressure, HR = heart rate, RA pacing = right atrial pacing, during HG = during handgrip. The closed circles represent mean values of group 1, open circles group 2, and closed squares group 3. [Reprinted from Amende, Krayenbuehl, Rutishauser & Wirz (27), with the permission of the publishers.]

whatever its esthetic benefits for developing skeletal muscle size, from a cardiological viewpoint isometric exercise is a relatively useless form of physical training and should not be recommended as a substitution for dynamic exercise. Indeed, it remains possible that intense isometric exercise should be avoided in certain conditions. Although static or isometric exercise of various types and intensities is performed many times daily during normal living, the activity is usually of insufficient intensity and duration to be harmful to patients with cardiovascular disease. Nevertheless, there are many anecdotal reports of untoward events occurring during heavy static exercise such as changing a tire, shoveling snow, or straining to open a stuck window. In patients with depressed cardiac function, the increase in arterial pressure may cause marked elevations of left ventricular end-diastolic pressure and ensuing pulmonary congestion. Also the increases in pressure load, heart rate, and contractile state serve to cause a marked increase in the oxygen demands of the left ventricle. This is especially harmful, of course, in patients with coronary heart disease.

Even though isometric exercise has been advocated as treatment in hypertension (70), it would appear that this type of activity could be potentially harmful in hypertensive patients. Studies of the cardiovascular responses to sustained handgrip in hypertensive patients have shown that systolic, diastolic, and mean arterial pressure increased markedly with moderate increases in heart rate and cardiac output (71, 72). The pressure levels reached by the hypertensive patients are much higher since they start from a higher resting pressure. Ewing et al (72) found two types of hemodynamic responses during handgrip in hypertensive patients. All showed marked elevations in arterial pressure but some demonstrated an increase in cardiac output with little change in peripheral vascular resistance and others an increase in peripheral vascular resistance with little change in cardiac output. The latter group had evidence of left ventricular hypertrophy and presumed decreased left ventricular performance. Although well-documented reports are rare, it seems likely that the high pressures that occur during static exercise in hypertensive patients may have clinical importance. Such pressure elevations could theoretically precipitate cerebrovascular hemorrhage or left ventricular failure in susceptible patients.

In summary, static exercise in patients, as in normal individuals, leads to hypertension and tachycardia with a resultant increase in myocardial oxygen utilization. This stress may be especially detrimental to patients with already compromised cardiovascular reserve. It would seem prudent, therefore, at the present time to advise patients with coronary heart disease or hypertension to avoid unsupervised isometric exertion.

ACKNOWLEDGMENTS

The authors are grateful to Elinor Reinmiller for her bibliographic assistance, to Margaret Wildenthal for translation of foreign papers, and to Pat Wells for secretarial assistance.

Literature Cited

1. Lind, A. R., McNicol, G. W., Donald, K. W. 1966. In *Physical Activity in Health and Disease,* ed. K. Evang, K. L. Andersen, 38–63. Oslo:Universitetsforlaget
2. Nutter, D. O., Schlant, R. C., Hurst, J. W. 1972. *Mod. Conc. Cardiovas. Dis.* 41:11–15
3. Lindhard, J. 1920. *Skand. Arch. Physiol.* 40:145–95
4. Alam, M., Smirk, F. H. 1937. *J. Physiol. London* 89:372–83
5. Alam, M., Smirk, F. H. 1938. *J. Physiol. London* 92:167–77
6. Asmussen, E., Hansen, E. 1938. *Skand. Arch. Physiol.* 78:283–303
7. Tuttle, W. W., Horvath, S. M. 1957. *J. Appl. Physiol.* 10:294–96
8. Humphreys, P. W., Lind, A. R. 1963. *J. Physiol. London* 166:120–35
9. Lind, A. R., Taylor, S. H., Humphreys, P. W., Kennelly, B. M., Donald, K. W. 1964. *Clin. Sci.* 27:229–44
10. Staunton, H. P., Taylor, S. H., Donald, K. W. 1964. *Clin. Sci.* 27:283–91
11. Macdonald, H. R., Sapru, R. P., Taylor, S. H., Donald, K. W. 1966. *Am. J. Cardiol.* 18:333–43
12. Lind, A. R., McNicol, G. W. 1967. *J. Physiol. London* 192:575–93
13. Lind, A. R., McNicol, G. W. 1967. *J. Physiol. London* 192:595–607
14. Donald, K. W. et al 1967. *Circ. Res.* 20:I-15–I-30
15. Lind, A. R., McNicol, G. W., Bruce, R. A., Macdonald, H. R., Donald, K. W. 1968. *Clin. Sci.* 35:45–53
16. Lind, A. R., McNicol, G. W. 1968. *J. Appl. Physiol.* 25:261–67
17. Muir, A. L., Donald, K. W. 1970. *Proc. Roy. Soc. London* 63:203–4
18. Freyschuss, U. 1970. *Acta Physiol. Scand. Suppl.* 342:1–63
19. Hirche, H., Raff, W. K., Grün, D. 1970. *Pfluegers Arch.* 314:97–112
20. Gray, S. D., Staub, N. C. 1967. *Am. J. Physiol.* 213:677–82
21. Karlsson, J., Ollander, B. 1972. *Acta Physiol. Scand.* 86:309–14
22. Ahlborg, B. et al 1972. *J. Appl Physiol.* 33:224—28
23. Mullins, C. B., Leshin, S. J., Mierzwiak, D. S., Matthews, O. A., Blomqvist, G. 1970. *Clin. Res.* 18:322
24. Helfant, R. H., deVilla, M. A., Meister, S. G. 1971. *Circulation* 44:982–93
25. Kivowitz, C. et al 1971. *Circulation* 44:994–1002
26. Krayenbuehl, H. P., Rutishauser, W., Schoenbeck, M., Amende I. 1972. *Am. J. Cardiol.* 29:323–30
27. Amende, I., Krayenbuehl, H. P., Rutishauser, W., Wirz, P. 1972. *Brit. Heart J.* 34:688–95
28. Mullins, C. B., Blomqvist, G. 1973. *Tex. Med.* 69:53–58
29. Fisher, M. L., Nutter, D. O., Jacobs, W., Schlant, R. C. 1973. *Brit. Heart J.* 35:422–32
30. Jackson, D. H., Reeves, T. J., Sheffield, L. T., Burdeshaw, J. 1973. *Am. J. Cardiol.* 31:344–50
31. Bergman, S. A. Jr., Campbell, J. K., Wildenthal, K. 1972. *J. Appl. Physiol.* 33:27–31
32. Cunningham, D. J. C., Strange Petersen, E., Peto, R., Pickering, T. G., Sleight, P. 1972. *Acta Physiol. Scand.* 86:444–55
33. Petro, J. K., Hollander, A. P., Bouman, L. N. 1970. *J. Appl. Physiol.* 29:794–98
34. Paulev, P. E. 1971. *J. Appl. Physiol.* 30:165–72
35. Borst, C., Hollander, A. P., Bouman, L. N. 1972. *J. Appl. Physiol.* 32:70–77
36. Krogh, A., Lindhard, J. 1913. *J. Physiol. London* 47:112–36
37. Asmussen, E., Christensen, E. H., Nielsen, M. 1940. *Skand. Arch. Physiol.* 83:181–87
38. Asmussen, E., Nielsen, M., Wieth-Pedersen, G. 1943. *Acta Physiol. Scand.* 6:353–58
39. Ochwadt, B., Bücherl, E., Kreuzer, H., Loeschcke, H. H. 1959. *Pfluegers Arch.* 269:613–21
40. Asmussen, E., Johansen, S. H., Jorgensen, M., Nielsen, M. 1965. *Acta Physiol. Scand.* 63:343–50
41. McCloskey, D. I., Matthews, P. B. C., Mitchell, J. H. 1972. *J. Appl. Physiol.* 33:623–26
42. McCloskey, D. I., Mitchell, J. H. 1972. *J. Physiol. London* 224:173–86
43. Pérez-González, J. F., Coote, J. H. 1972. *Am. J. Physiol.* 222:138–43
44. Goodwin, G. M., McCloskey, D. I., Mitchell, J. H. 1972. *J. Physiol. London* 226:173–90
45. Kao, F. F., Ray, L. H. 1954. *Am. J. Physiol.* 179:249–54
46. Kao, F. F., Lahiri, S., Wang, C., Mei, S. S. 1967. *Circ. Res.* 20:I–179–I–191
47. Coote, J. H., Hilton, S. M., Perez-Gonzalez, J. F. 1971. *J. Physiol. London* 215:789–804
48. Gordon, G. 1943. *J. Physiol. London* 102:95–107

49. Johansson, B. 1962. *Acta Physiol. Scand.* 57 (Suppl. 198):1–91
50. Mitchell, J. H., Mierzwiak, D. S., Wildenthal, K., Willis, W. D. Jr., Smith, A. M. 1968. *Circ. Res.* 22:507–16
51. Coote, J. H., Perez-Gonzalez, J. F. 1970. *J. Physiol. London* 208:261–78
52. Wildenthal, K., Mierzwiak, D. S., Skinner, N. S. Jr., Mitchell, J. H. 1968. *Am. J. Physiol.* 215:542–48
53. Kjellmer, I. 1961. *Med. Exp.* 5:56–60
54. Laurell, H., Pernow, B. 1966. *Acta Physiol. Scand.* 66:241–242
55. Saltin, B. et al 1968. *Circulation* 38:VII-1–VII-78
56. Hník, P., Hudlická, O., Kučera, J., Payne, R. 1969. *Am. J. Physiol.* 217:1451–57
57. Wildenthal, K., Mierzwiak, D. S., Mitchell, J. H. 1969. *Am. J. Physiol.* 216:898–904
58. Mellander, S. et al 1967. *Angiologica* 4:310–22
59. Lasser, R. P., Schoenfeld, M. R., Allen, D. F., Friedberg, C. K. 1960. *Circ. Res.* 8:913–19
60. Stegemann, J., Ulmer, H. V., Böning, D. 1967. *Pfluegers Arch.* 293:155–64
61. Houston, J. D., Atkins, J. M., Blomqvist, G. 1970. *Clin. Res.* 18:70
62. Siegel, W., Gilbert, C. A., Nutter, D. O., Schlant, R. C., Hurst, J. W. 1972. *Am. J. Cardiol.* 30:48–54
63. Matthews, O. A., Blomqvist, C. G., Cohen, L. S., Mullins, C. B. 1970. *Circulation* 42:III-31
64. Mösseler, U., Schlepper, M. 1971. *Z. Kreislaufforsch.* 60:1067–73
65. Payne, R. M., Horwitz, L. D., Mullins, C. B. 1973. *Am. J. Cardiol.* 31:428–33
66. Krayenbuehl, H. P., Rutishauser, W., Wirz, P., Amende, I., Mehmel, H. 1973. *Am. J. Cardiol.* 31:415–27
67. Jackson, D. H. 1970. *Ala. J. Med. Sci.* 7:310–12
68. Lind, A. R. 1970. *Circulation* 41:173–76
69. Müller, E. A. 1965. *Ergonomics* 8:409–24
70. Kiveloff, B., Huber, O. 1971. *J. Am. Geriat. Soc.* 19:1006–12
71. Hoel, B. L., Lorentsen, E., Lund-Larsen, P. G. 1970. *Acta Med. Scand.* 188:491–95
72. Ewing, D. J., Irving, J. B., Kerr, F., Kirby, B. J. 1973. *Brit. Heart J.* 35:413–21

Copyright 1974. All rights reserved

CONTROL OF PLATELET PRODUCTION[1]

◆7120

Laurence A. Harker, M.D.
Division of Hematology, University of Washington, Seattle, Washington

The constancy of the circulating platelet concentration and the reciprocal changes in platelet production that follow alteration in this concentration reflect a compensatory mechanism that plays a major role in disorders of the platelet-megakaryocyte system (1–13). Since the mechanisms controlling platelet production modify each phase of megakaryocyte development, it is appropriate to review normal megakaryocytopoiesis.

NORMAL THROMBOPOIESIS

The normal process of producing platelets involves the development of the megakaryocyte through four overlapping phases: (*a*) formation of identifiable polyploid cells from morphologically unrecognizable mononuclear precursors; (*b*) nuclear replication within each cell (endoreduplication); (*c*) cytoplasmic maturation and demarcation into platelet subunits; and (*d*) platelet release into the circulation (Figure 1).

Initial Formation and Nuclear Replication

Megakaryocytes arise from diploid precursor cells which are derived from uncommitted stem cells (14, 15). At present, megakaryocytes can only be identified after they become polyploid. Since the process of nuclear replication within a common cytoplasm proceeds synchronously, ploidy doubles with each mitotic event until the full polyploid complement is achieved (16–19). Endoproliferation occurs early in development, i.e. within the first day or two, as illustrated by the megakaryoblast. In this cell, despite its sparse, basophilic, nongranular cytoplasm and irregular,

---

[1] Supported by research grants (HL-11775 and HL-06242) from the National Institutes of Health. A portion of this work was conducted on Clinical Research Center facilities at the University of Washington (NIH grants FR-37 and RR-133). This investigation was done during the tenure of an established investigatorship of the American Heart Association.

immature nucleus, DNA synthesis is nearly complete. The chromosomal material in mature megakaryocytes assumes a lobular configuration, each lobe being approximately diploid (2 N) (9, 12, 20). In normal marrow about two thirds of the megakaryocytes are 16 N (8 nuclear lobes); about a fourth are 32 N (16-lobed); and the remainder are 8 N (4-lobed).

Cytoplasmic Maturation and Platelet Release

Although megakaryocytic organelles may be present in DNA-synthesizing cells (21), cytoplasmic maturation largely follows proliferation (18, 19). Maturation is characterized by the elaboration of specific granules from the Golgi apparatus that eventually fill the cytoplasm completely except for a thin peripheral, amorphous rim. Simultaneously, the cytoplasm becomes demarcated into platelet subunits by a surface-communicating plasma membrane system (Figure 2) (22–25). Cytoplasmic maturation requires about three days and is manifested in stained preparations by the appearance of azurophilic granulation. About a fourth of the megakaryocytes in normal marrow have no apparent granulation; a fourth show partial granulation; and those of the remaining half appear fully granulated. The amount of cytoplasm accumulated by each megakaryocyte during maturation is predetermined by its ploidy (9, 12, 13). The megakaryocyte can be likened to a clone of cells undergoing common proliferation and maturation that is composed of 2^n diploid cell equivalents or "nuclear units," i.e. nuclei and the associated cytoplasmic complement (9).

The average platelet size remains relatively constant over a wide range of pathophysiologic states (26); therefore, it follows that the number of platelets produced by each megakaryocyte nuclear unit remains unchanged at about 150–200. The marrow substrate available for platelet production is represented by the mass of

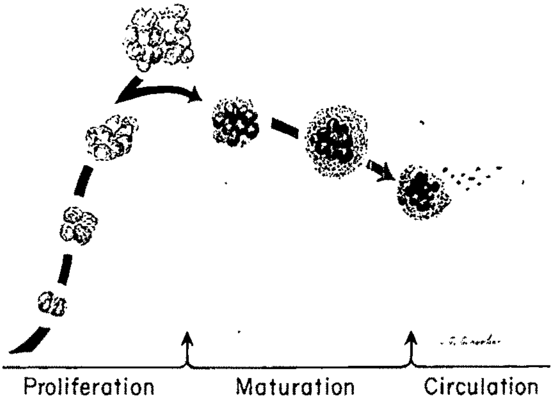


Figure 1 Megakaryocyte development.

megakaryocytic cytoplasm; this quantity is expressed as the product of the mean megakaryocyte cytoplasmic volume and the total number of megakaryocytes (12).

Platelet release initially involves emergence of the preformed platelet mass from the megakaryocyte through marrow sinusoids into the circulation. Cytoplasmic fragmentation into platelets requires at least several hours (27, 28). The residual bare nuclear material is then removed by reticuloendothelial cells.

Platelet Kinetics

Human platelets circulate as cytoplasmic discs at a concentration of 250,000 per μl ± 40,000, with an average volume of about 10 mm^3 and a finite lifespan of 9.5 days ± 0.6 (12, 29). In normal subjects, two thirds of the total platelets are in the general circulation while the remaining third form a splenic pool that exchanges freely with the general circulation. The distribution between the systemic and the splenic compartments can be estimated by determining the proportion of ^{51}Cr-labeled platelets remaining in circulation immediately after infusion. In asplenic individuals, nearly 100% of the labeled platelets are recovered. Conversely, splenomegaly is associated with low recovery due to a marked increase in splenic pooling involving as much as 85–90% of the total body platelets (30, 31). The linear disappearance pattern of ^{51}Cr-labeled platelets primarily reflects a finite lifespan that is largely independent of any ongoing platelet consumption in support of normal

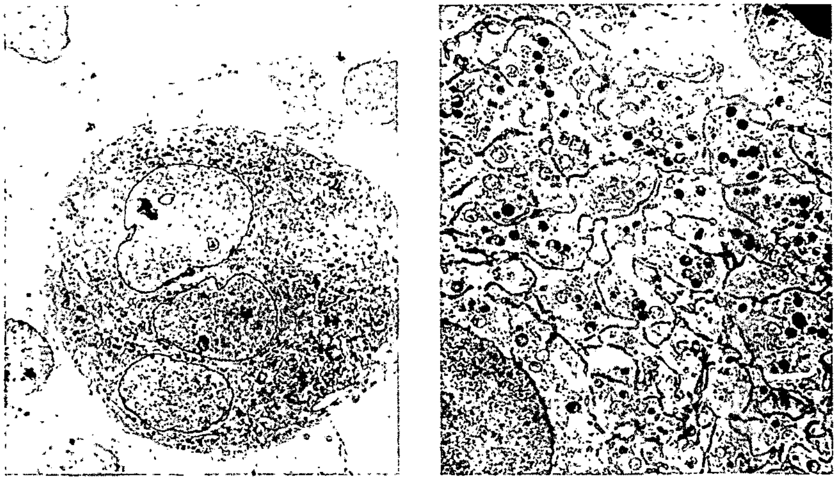


Figure 2 Ultrastructural view of cytoplasmic maturation. (*left*) Rat megakaryocyte showing the nucleus and the cytoplasm containing granular and membranous elements with a peripheral shell relatively free of organelles (uranyl acetate and lead stain × 2000). (*right*) Higher power view of developing platelet subunits showing granules, mitochondria, and demarcation membranes (uranyl acetate and lead stain × 20,000).

hemostasis (12, 29). Platelet turnover, i.e. platelet count divided by platelet survival, reflects the rate of removal from the circulation. In the steady state, however, platelet turnover is also a measure of viable platelet delivery from the marrow to the circulation; it is therefore synonymous with effective thrombopoiesis (12, 32, 33).

The generating mass of megakaryocytic cytoplasm is directly reflected as circulating platelets (Figure 3). This relationship can also be expressed as the rate at which platelets are produced per nuclear unit, i.e. 50 platelets per nuclear unit per day in normal individuals. Defects in megakaryocyte development or intramedullary destruction result in impaired delivery of viable platelets, i.e. ineffective thrombopoiesis, identified as a disparity between megakaryocytic mass and platelet turnover (12).

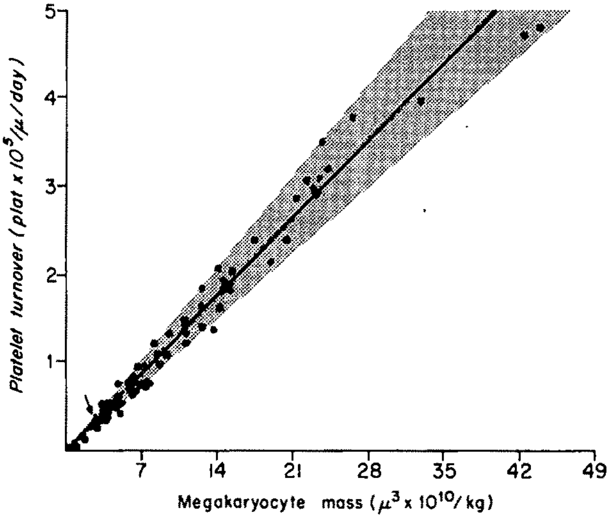


Figure 3 Megakaryocyte mass is directly reflected as platelet turnover (the circulating product). Normal values are shown by arrow. Shaded area includes 95% confidence limits. (Reprinted with permission from *J. Clin. Invest.* 1969. 48:963.)

STIMULATED THROMBOPOIESIS

Severe, sustained thrombocytopenia induces a two- to fourfold increase in platelet production within several days. Production can expand to as much as six to ten times normal in response to chronic thrombocytopenia. This compensatory response represents the summation of changes in all phases of megakaryocyte development. In the following paragraphs, the relative contribution and the timing of each component of this homeostatic response are analyzed.

Platelet Release

Immediately after an acute, severe thrombocytopenic stimulus, a modest number of readily available platelets are mobilized over several hours from mature megakaryocytes (Table 1). This mass of prematurely released platelets is equivalent to about one day's expected platelet production; it is characterized morphologically by the presence of ribosomes (28) and functionally by increased hemostatic competence, e.g. enhanced spreading on glass surfaces and shortened bleeding time (34–36). No change in the volume of these platelets is demonstrable when adequate sizing techniques are used (26, 37). The release of these rapidly mobilized platelets is accompanied by a reciprocal loss of mature megakaryocyte mass as evidenced by the shift of ^{35}S-labeled megakaryocyte cytoplasm to circulating platelets (Table 1).

Cytoplasmic Maturation

The time required for megakaryocyte cytoplasmic maturation has been assessed by cytoplasmic labels such as ^{35}S-sulfate and ^{75}Se-selenomethionine (7, 9, 38–40). Measurements of the marrow transit time of ^{35}S-sulfate have shown that sustained platelet depletion shortens the time of maturation (40) (Figure 4). Studies using a nuclear label and megakaryocyte sizing also indicate a decreased maturation time in association with thrombocytopenia (41). Furthermore, there is a decrease in the proportion of megakaryocytes showing no cytoplasmic granulation with severe

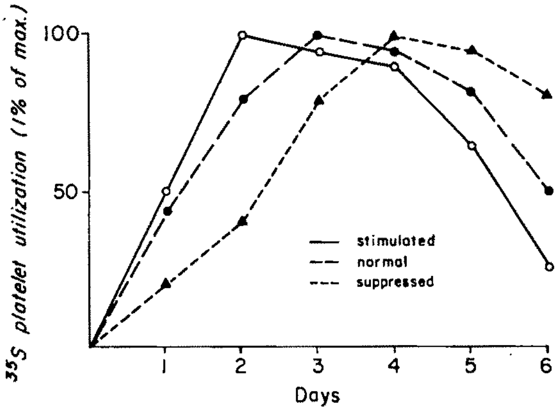


Figure 4 Comparison of platelet incorporation of ^{35}S-sulfate in animals with normal, 4-day stimulated, and 4-day suppressed thrombopoiesis. Each point represents the mean of ten animals. Stimulated production involves an increase in the rate of appearance; the converse occurs as platelet production is suppressed. Differences on the second day are significant at the 5% level; suppressed thrombopoiesis also incorporates significantly less ^{35}S-sulfate on the third day (at 5% level). After the fourth day differences are again significant at the 5% level.

Table 1 Stimulated platelet release[a]

	Megakaryocytes			Platelets			
	Total Number X 10^6/kg	Mean Volume (μm^3)	Total Mass ($\mu m^3 \times 10^9$)	Concentration Platelets X $10^6/\mu l$	Mean Volume (μm^3)	Total Mass ($\mu m^3 \times 10^9$)	^{35}S Platelet Activity cpm/10^6 plat
Baseline	11.0 ± 1.9	18,000	49.5 ± 3.5	1.27 ± 0.13	4.5	74.3	1.8 ± 0.3
Post Exchange							
Immediate	10.7 ± 1.7	17,000	45.5 ± 3.4	0.07 ± 0.01	4.6	4.2	2.1 ± 0.5
4-Hr	10.3 ± 1.8	13,000	33.5 ± 2.0	0.30 ± 0.05	4.5	17.6	14.1 ± 1.7
Difference	~0	−4,000	−12.0	+0.23	~0	+13.4	+12.0

[a] Previously splenectomized rats that had been injected with ^{35}S two days before were exchange transfused rapidly with platelet-poor blood until the peripheral platelet count was reduced to about 5% of baseline count. Megakaryocyte mass, and platelet count, volume, mass and ^{35}S specific activity were compared immediately after platelet depletion and 4 hr later when the platelet count had risen to 300,000 plat/μl. Mean megakaryocyte volume was decreased and platelet size was unchanged. The increase in the circulating platelet mass was equivalent to the decrease in megakaryocyte mass. The shift of mature ^{35}S-labeled megakaryocyte cytoplasm to the peripheral blood as circulating platelets is confirmed by the appearance of ^{35}S in platelets. Each value is the mean of six animals.

thrombocytopenia; this change has been interpreted as reflecting decreased maturation time (42), although an extended proliferative phase (to accommodate additional endomitosis) may also be contributory.

Endoreduplication

Within hours of an acute thrombocytopenic stimulus, the young, DNA-synthesizing megakaryocytes undergo, on the average, one additional endomitosis (9, 10, 13, 19). The doubling of both ploidy and cytoplasmic mass becomes fully manifest in the marrow after the second or third day (Figure 5), while the resultant increased platelet production follows one or two days later. Parallel changes in endomitosis and platelet production have been documented by ^{35}S-platelet utilization and platelet turnover measurements (9, 12) (Figure 6). The stimulus for additional endoreduplication appears to be determined by the severity of the thrombocytopenia, as shown by the inverse, first-order relationship between the circulating platelet count, megakaryocyte volume, and nuclear lobe number (Figure 7). This association may be mathematically represented: Average nuclear lobe number = 36 – 5 log (platelet count/μl). Some immune thrombocytopenic states appear to have an additional population of immature, low-ploidy megakaryocytes that may not be recognized in marrow sections; their significance is uncertain (43).

Megakaryocyte Formation

Chronic thrombocytopenia increases the number of megakaryocytes by enhancing the rate of influx from the diploid precursor pool (5, 9, 13, 15, 40). This process of

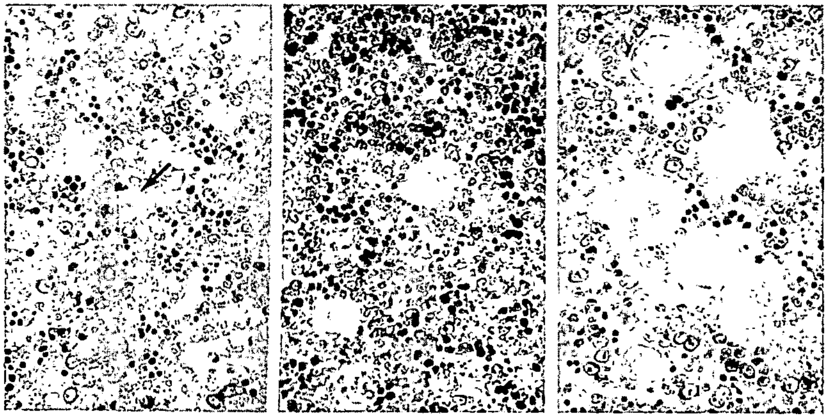


Figure 5 Comparative photomicrographs of representative bone marrow sections from animals with normal (*center panel*), suppressed (*left panel*), and stimulated (*right panel*) thrombopoiesis taken at a magnification of 320. The change in size and number of megakaryocytes is apparent. (Reproduced with permission from *J. Clin. Invest.* 1968. 47:468.)

expanding megakaryocyte number, while initially modest, progresses at a gradual, steady increment, beginning within a day or two of stimulation and requiring weeks to achieve a maximum rate, which is about 4–5 times greater than normal (9, 12, 19, 41). Although the capacity to enlarge megakaryocyte number is delayed, it is ultimately the most important factor determining the quantitative thrombopoietic response.

Mobilization of the splenic platelet pool into the general circulation represents a sizable potential source of platelets that is immediately, although transiently, available under adrenergic stimulation. The brevity of the response and the intensity of the required stimulus suggest that this mechanism probably provides no useful

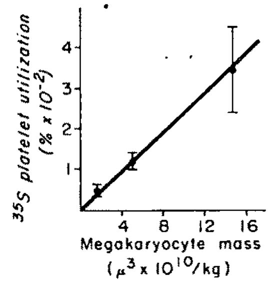


Figure 6 Relationship between radiosulfur platelet incorporation and megakaryocyte mass. Maximal mean ^{35}S-platelet utilization values ± 1 SD from suppressed, normal, and stimulated thrombopoiesis are compared with mean megakaryocyte mass measurements on animals comparably treated. (Reproduced with permission from *Am. J. Physiol.* 1970. 218:1376.)

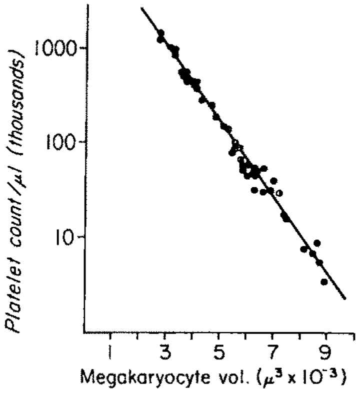


Figure 7 Inverse first-order relationship between megakaryocyte volume and platelet count.

hemostatic compensation, as further evidenced by the constancy of normal splenic pool size in a wide range of pathological thrombocytopenias (12, 30, 42). The only documented exception is the immediate postoperative period, when adrenergic stimulation appears to be sufficiently prolonged to sustain a shift out of the splenic pool. The peripheral platelet count is thus supported for the first day or two until increased thrombopoiesis provides for the increased requirements of wound repair (30, 44).

In summary, the compensatory sequence induced by acute thrombocytopenia includes premature release of platelets within hours, increased rate of cytoplasmic maturation during the first day, increased production of platelets per megakaryocyte due to additional endomitosis on the second and third day, and subsequently, an increase in the rate of megakaryocyte formation. On rare occasions, whenever thrombocytopenia is associated with a strong sustained adrenergic stimulus, mobilization of the splenic platelet pool can occur within minutes.

SUPPRESSED THROMBOPOIESIS

Effect of Platelet Hypertransfusion

Platelet hypertransfusion is an effective means of reducing endogenous platelet production (3, 7–9, 12, 40, 45). The changes produced in megakaryocytopoiesis by platelet hypertransfusion are the converse of the findings in stimulated thrombopoiesis, i.e. reduction in the rate of cytoplasmic maturation (Figure 4), decrease in megakaryocyte size and ploidy (Figures 4, 5, 7), and a decrease in megakaryocyte number (9, 12, 40).

Sustained thrombocytosis is essential for decreasing endogenous thrombopoiesis, since injecting comparable numbers of intact but nonviable (cold-injured) platelets does not suppress megakaryocytopoiesis (9, 40). However, thrombopoiesis cannot be reduced below about 20% of normal (Figure 8), even by hypertransfusion up to ten times the normal circulating count and for periods as long as four weeks (37,

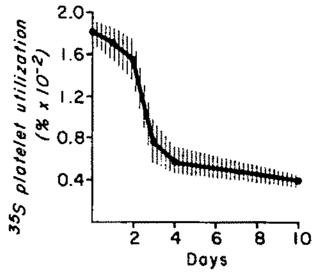


Figure 8 Suppressive effect of platelet hypertransfusion of thrombopoiesis in splenectomized animals. ^{35}S was injected after increasing periods of induced thrombocytosis (0-10 days) and radiosulfur platelet utilization was measured three days later. (Reproduced with permission from *Am. J. Physiol.* 1970. 218:1376.)

40). Apparently there is an intrinsic basal level of megakaryocytopoiesis that is not subject to physiological regulation.

Isotope Measurements

Isotopic measurements of platelet production are particularly valuable in assessing suppressed thrombopoiesis since platelet counts and platelet turnover are not meaningful in this experimental situation, and megakaryocyte mass determinations are excessively demanding for assay purposes. Injected radiosulfur or ^{75}selenomethionine is irreversibly incorporated into the cytoplasm of maturing marrow megakaryocytes and subsequently appears in the circulation bound to the released platelets (Figure 9) (8, 38–40, 46, 47). Platelets already in circulation do not incorporate the label. When the incorporation of the label into platelets is expressed as a percentage of the injected dose, the figures obtained are in agreement with measurements of megakaryocyte mass in animals with normal, stimulated, and suppressed thrombopoiesis (Figure 6).

It is of some concern that isotopically labeled sulfur or selenomethionine is incorporated into many body tissues after injection, so that only a small proportion appears in developing platelets. This lack of specificity is a probable source of error in interpreting results of isotope incorporation into platelets.

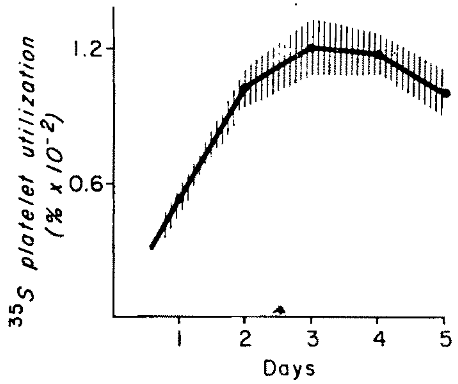


Figure 9 Normal pattern of platelet incorporation of ^{35}S in rats. Each point represents mean of ten animals; ±1 SD is shown by shaded area. (Reproduced with permission from *Am. J. Physiol.* 1970. 218:1376.)

REGULATORY MECHANISMS

Thrombopoietic Stimulating Factors

The physiologic data are consistent with the view that an intrinsic basal rate of megakaryocytopoiesis becomes stimulated in order to meet the requirements for circulating platelets: a modest increase is needed to meet normal demands, and marked increases occur in response to platelet depletion.

Similarities between thrombopoiesis and erythropoiesis prompted early workers to postulate a humoral stimulator analogous to erythropoietin and to designate it "thrombopoietin" (48). Confirmation of this hypothesis has proved technically difficult. Numerous investigators have observed a rise in the platelet count of animals following the injection of serum, plasma, urine, or various biological extracts (49–59), and they have understandably attributed the effect to thrombopoietic activity in the material injected. However, it is now apparent that these results cannot be differentiated from a nonspecific endogenous thrombopoietic response (60), and assays using normal animals are thereby compromised (61–64). In order to make meaningful measurements of stimulated platelet production, endogenous thrombopoiesis must be suppressed by platelet hypertransfusion before injecting the material being tested. The thrombopoietic effect of the test material is then measured as incorporation of ^{35}S or ^{75}selenomethionine into platelets. Such experiments (Figure 10) have shown that when plasma from one hypertransfused ("suppressed") animal is injected into another, there is no change in isotope incorporation. However, plasma from normal animals causes more than a twofold increase, and plasma from animals with thrombocytopenia produces nearly a fourfold increase. The results of such studies indicate the presence of thrombopoietic stimulating activity in plasma of normal animals, which becomes augmented during thrombocytopenia and is undetectable after platelet hypertransfusion (40, 45). Such data reinforce the analogy with erythropoietin.

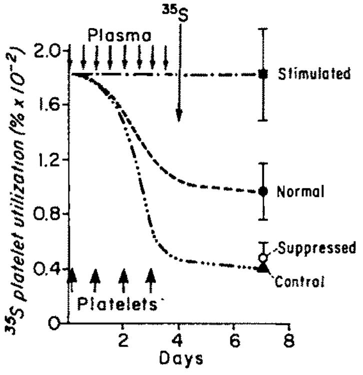


Figure 10 Thrombopoietic-stimulating activity in plasma is assayed in animals with suppressed endogenous thrombopoiesis using ^{35}S-sulfate platelet utilization to measure platelet production. Plasma from animals with suppressed thrombopoiesis has no significant effect on suppressed controls. Plasma from animals with stimulated platelet production produces a greater than threefold increase in ^{35}S-sulfate platelet incorporation over the baseline value. (Reproduced with permission from *Am. J. Physiol.* 1970. 218:1376.)

The technical problems associated with a hypertransfusion assay system are considerable. Platelet hypertransfusion imposes heavy burdens of time, animals, and expense that prevent investigators from carrying out large numbers of determinations, as would be necessary for example to perform plasma fractionation experiments. Recent work using ammonium sulfate-precipitated plasma fractions shows that the thrombopoietic activity is contained in the 60–80% precipitable fraction (64), but progress will be slow until more efficient assay systems are devised. Efforts are currently being directed towards in vitro techniques such as megakaryocyte culture (65) and immune techniques (66). These methods will obviously require extensive development.

A child with congenital, severe thrombocytopenia that responded transiently to infusions of normal plasma has been reported to represent a hereditary deficiency of thrombopoietin (55, 58). This interpretation seems unlikely. In the first place, rather than showing a decreased number of small megakaryocytes that were otherwise morphologically normal, as would be expected from studies of suppressed endogenous thrombopoiesis (9, 12), the patient's marrow had an abundance of rather bizarre immature megakaryocytes—suggesting a major component of ineffective thrombopoiesis as has been found in other hereditary thrombocytopenias (67). Secondly, a single infusion of normal plasma amounting to 10% of the child's plasma volume could not be expected to provide sufficient "thrombopoietin" to produce a circulating platelet count three to four times greater than normal. It is more likely that the patient has thrombocytopenia on the basis of ineffective thrombopoiesis that is related to a hereditary deficiency of some maturation factor contained in normal plasma—analogous to vitamin B_{12}-deficient thrombopoiesis. (12)

The Sensor Mechanism

The existing data indicate that thrombopoiesis is regulated to maintain the concentration of circulating platelets within narrow limits, probably by some type of feedback mechanism. Although it seems physiologically more sound to propose some aspect of platelet function as the critical determinant (analogous to oxygen-delivery regulation of erythropoiesis), maintenance of normal platelet counts in patients with hereditary or acquired disorders of platelet function, e.g. thrombasthenia, is rather compelling evidence against a functionally related sensing system (68). Additional relevant evidence consists of the converse observation that thrombopoiesis remains increased severalfold over normal in patients with normal platelet function who have increased platelet destruction with or without thrombocytopenia (36, 42). It is of course possible that an unrecognized function of platelets may yet prove to be the discriminating factor.

Alternatively, the circulating platelet mass has been proposed as the regulatory indicator (69, 70). While this possibility is consistent with the fact that patients with hereditary macrothrombocytes have decreased platelet counts (71), defective megakaryocytopoiesis in these patients seems an equally acceptable explanation (67). In addition, thrombopoiesis in patients with a greatly increased total platelet mass due to increased splenic pooling appears to be regulated in response to the circulating platelet level rather than to the total platelet mass (12, 72). Removal of thrombopoietic stimulator by circulating platelets is the postulated means whereby platelet mass might regulate the thrombopoietic activity (8). This hypothesis seems unlikely since a severalfold increase in the passage of platelets through blood (i.e. elevated platelet turnover) is by definition associated with increased thrombopoiesis (12), and conversely, platelet production remains normal despite the infusion of large numbers of intact but short-lived platelets (9).

The Stimulator Mechanism

The features of stimulated thrombopoiesis can be largely explained by a single humoral agent that stimulates both the rate and number of mitoses. Mitogenic stimulation of early diploid precursor cells would increase the number of megakaryocytes formed, while stimulation of megakaryoblasts would increase the number of endomitoses per cell and thereby cell size. Alternatively, it has been suggested that action of the stimulator is confined to increasing the rate of mitosis while the number of maturation mitoses is limited by the action of some normal constituent of megakaryocytic cytoplasm (73). In this formulation, premature release and shortened cytoplasmic maturation time represent passive cytoplasmic shedding from fully mature megakaryocytes into platelet-depleted blood.

Such postulated mechanisms are at present little more than speculative, and they become even less convincing when disorders of thrombocytosis are examined. In reactive thrombocytosis, for example, megakaryocyte ploidy decreases in accord with prediction based on the level of circulating platelets (Figure 11). Megakaryocyte number, on the other hand, is greatly increased. The disparity between megakaryocyte size and number has prompted the suggestion that there are two thrombopoietic stimulators—the first increasing the number of endomitoses (maturation divisions) and the second increasing the rate of megakaryocyte formation (replicative divisions). These two stimulators are postulated to act together in the presence of thrombocytopenia, but both are capable of acting independently (40). In this scheme, the enlarged megakaryocytes of thrombocythemia (idiopathic thrombocytosis) are thought to result from autonomous endoreduplication of an abnormal clone of megakaryocytes that is not responsive to physiologic regulation (Figure 12), and thrombopoietic stimulating factor(s) are markedly suppressed because of the elevated platelet level. Destruction of the abnormal megakaryocytes by cytotoxic agents, e.g., busulfan, would cause a fall in the platelet count with a resultant increase in the circulating stimulator (Figure 12). Normally responsive megakaryocytes would then repopulate the marrow, maintaining the platelet count until overgrowth by the abnormal clone reestablished the initial state. This formulation, which is at variance with reports of elevated "thrombopoietin" levels in thrombocythemia (6, 74), awaits the availability of a reliable thrombopoietic factor assay for critical testing.

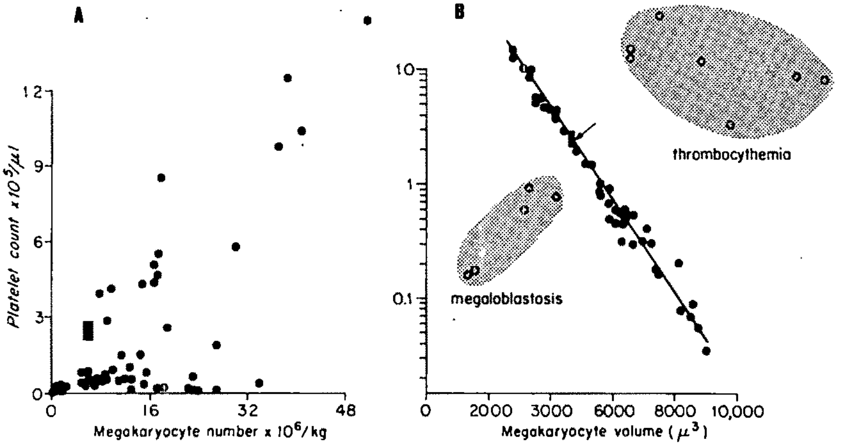


Figure 11 The megakaryocyte response to the circulating platelet count is shown in relationship to megakaryocyte number (*A*) and mean megakaryocyte volume (*B*). While no correlation was apparent between megakaryocyte number and the platelet count, an inverse first-order relationship was apparent between mean megakaryocyte volume and the circulating platelet count. The normal mean value ±1 SD appears as a black rectangle located by the arrow. In two disorders, megaloblastosis and thrombocythemia, the usual relationship between the platelet count and megakaryocyte volume was disturbed. In megaloblastosis the limitation of nuclear replication restricted endomitosis, and hence volume, despite thrombocytopenia. In contrast, thrombocythemia was characterized by increased endomitosis (and therefore volume) in the presence of high platelet counts, which is evidence of autonomous proliferation. (Reproduced with permission from *J. Clin. Invest.* 1969. 48:972.)

OTHER THROMBOPOIETIC EFFECTS

Role of the Spleen

Finding an inverse association between spleen size and platelet levels in some patients suggested to early workers that the spleen might control the platelet count either by splenic destruction of platelets (75) or by suppression of thrombopoiesis through a humoral inhibitory effect, i.e. "hypersplenism" (76). The subsequent controversy between these possibilities was seemingly resolved by later reports claiming that the spleen contained both stimulatory and inhibitory factors (77, 78). Currently, neither clinical nor critical experimental evidence directly support the notion of a humoral regulatory role of the spleen since it is now known that the beneficial effect of splenectomy in idiopathic thrombocytopenic purpura is due to removal of a major site of platelet destruction, and "hypersplenic" thrombocytopenia is caused by pooling of platelets in the enlarged spleen (30, 42). The transient thrombocytosis following traumatic splenectomy seems adequately explained by a

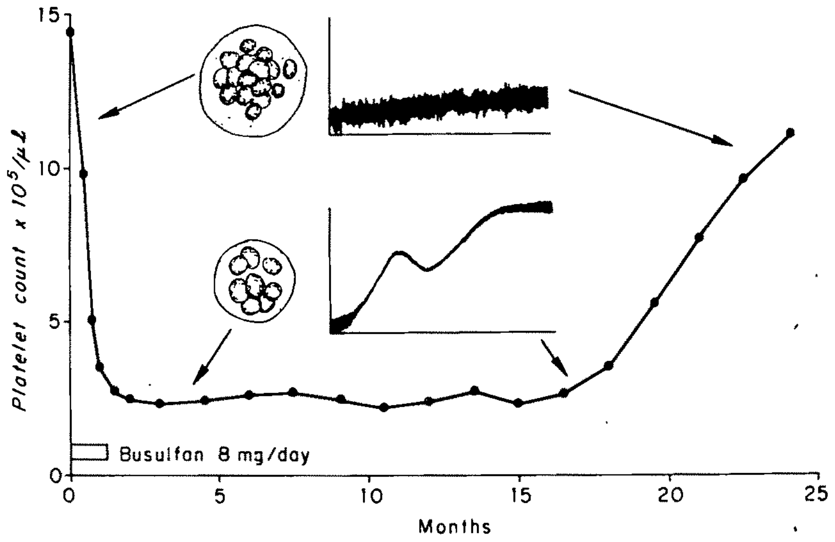


Figure 12 Busulfan therapy in thrombocythemic patient. The enlarged autonomous megakaryocytes of thrombocythemia produce increased numbers of defective platelets (no aggregation by epinepherine) which suppress endogenous thrombopoietic factor. Destruction of autonomous megakaryocytes by busulfan is thought to be followed by repopulation with normal megakaryocytes due to an elevation in the level of stimulating factors. Normal thrombopoiesis continues until the abnormal cells again overgrow the regulated megakaryocytes.

combination of the usual temporary post-operative increase in platelet production and removal of the normal splenic pool. For reasons that are unknown, however, splenectomy performed as a part of the therapy for hemolytic disease is not infrequently followed by thrombocytosis that persists for months or even indefinitely.

Relation to Erythropoietin

Although increased erythropoiesis may be associated with an elevated platelet count as suggested above (79–81), no correlation has been found with (*a*) thrombopoietic plasma activity (8, 55, 82), (*b*) hypoxia (83), or (*c*) polycythemia or nephrectomy (8). Large amounts of partially purified erythropoietin, however, have recently been shown to have measurable thrombopoietic activity (84). This apparent discrepancy remains to be resolved.

Other Hormones

Menstruating females reportedly show a cyclic reduction in their platelet counts, being most pronounced at or just before the onset of menses (85); an exaggeration of this phenomenon has been observed during normal pregnancy (86). The mechanism is presumed to involve decreased megakaryocytopoiesis, since estrogenic hormones cause severe megakaryocytic hypoplasia in dogs (87) and rabbits (88).

While androgenic hormones are not usually considered to affect platelet production, some aplastic patients with severe, threatening thrombocytopenia may show a modest but critical rise in their platelet counts following pharmacologic doses of androgens (37).

Large doses of corticosteroids for prolonged periods have been associated with decreased platelet counts in some patients with idiopathic thrombocytopenia (89). The frequency and relevance of these observations is not clear.

SUMMARY

On the basis of available data, it is concluded that platelet production in the marrow is regulated to meet the requirements for circulating platelets. It is presumed that the poorly characterized, humoral thrombopoietic activity demonstrated to be present in normal plasma and increased in "thrombocytopenic" plasma represents the regulatory stimulator of megakaryocytopoiesis, analogous to erythropoietin stimulation of erythropoiesis. The thrombopoietic stimulating factor is thought to act by increasing the rate of: (*a*) megakaryocyte formation; (*b*) megakaryocyte endomitosis; and (*c*) cytoplasmic maturation and release.

Literature Cited

1. Brecker, G., Schneiderman, M., Cronkite, E. P. 1953. *Am. J. Clin. Path.* 23: 15–26
2. Matter, M., Kartmann, J. R., Kautz, J., DeMarsh, Q. B., Finch, C. A. 1960. *Blood* 15:174–85
3. Cronkite, E. P., Bond, V. P., Fliedner, T. M., Paglia, D. A., Adamik, E. R. 1961. *Blood Platelets,* ed. S. A. Johnson, R. W. Monto, J. W. Rebuck, R. C. Horn Jr., 595–601. Boston: Little Brown
4. Odell, T. T. Jr., Kniseley, R. M. 1962. *Progress in Hematology,* ed. L. M. Tocantins, 3:203–17. New York: Grune & Stratton
5. Odell, T. T. Jr., McDonald, T. P., Asano, M. 1962. *Acta Haematol.* 27:171–79
6. Kelemen, E., Lehoczky, D., Cserhati, I., Kriza, F., Rak, K. 1963. *Acta Haematol.* 29:16–26
7. Odell, T. T. Jr., Jackson, C. W., Reiter, R. S. 1967. *Acta Haematol.* 38:34–42
8. deGabriele, G., Penington, D. G. 1967. *Brit. J. Haematol.* 13:202–9
9. Harker, L. A. 1968. *J. Clin. Invest.* 47:458–65
10. Ebbe, S., Stohlman, F. Jr., Overcash, J., Donovan, J., Howard, D. 1968. *Blood* 32: 383–92
11. Ebbe, S., Stohlman, F. Jr., Donovan, J., Overcash, J. 1968. *Blood* 32:787–95
12. Harker, L. A., Finch, C. A. 1969. *J. Clin. Invest.* 48:963–74
13. Pennington, D. G., Olsen, T. E. 1970. *Brit. J. Haematol.* 18:447
14. Till, J. E., McCulloch, E. A. 1961. *Radiat. Res.* 14:213
15. Ebbe, S., Stohlman, F. Jr. 1965. *Blood* 26:20–35
16. Garcia, A. M. 1964. *J. Cell Biol.* 20:342
17. Paulus, J. M. 1968. *Nouv. Rev. Fr. Hematol.* 8:394
18. Odell, T. T. Jr., Jackson, C. W. 1968. *Blood* 32:102
19. Odell, T. T. Jr., Jackson, C. W., Friday, T. J., Charsha, D. E. 1969. *Brit. J. Haematol.* 17:91
20. Japa, J. 1943. *Brit. J. Exp. Pathol.* 24:73
21. Paulus, J. M. 1970. *Blood* 35:298
22. Yamada, E. 1957. *Acta Anat.* 29:267
23. de Bruyn, P. P. H. 1964. *Z. Zellforsch. Mikrosk. Anat.* 64:111–18
24. Ham, S. S., Baker, B. L. 1964. *Anat. Rec.* 149:251
25. Behnke, O. 1968. *J. Ultrastruct. Res.* 24:412
26. Lander, H., von Behrens, W. E. 1970. *Proc. Aust. Soc. Med. Res.* 2:372

27. Kinosita, R., Ohno, S. 1951. *Henry Ford Hospital Symposium on Blood Platelets*, ed. S. A. Johnson et al, 611–16. Boston: Little Brown
28. Behnke, O. 1969. *J. Ultrastruct. Res.* 26:111
29. Abrahamsen, A. F. 1968. *Scand. J. Haematol. Suppl.* 3:7–53
30. Aster, R. J. 1966. *J. Clin. Invest.* 45:645–57
31. Penny, R., Rozenberg, M. C., Firkin, B. G. 1966. *Blood* 27:1–16
32. Mustard, J. F., Roswell, H. C., Murphy, E. A. 1966. *Brit. J. Haematol.* 12:1
33. Davey, M. G. 1966. *The Survival and Destruction of Human Platelets*, p. 99. Basel, Switzerland: Karger
34. Hirsh, J., Glynn, M. F., Mustard, J. F. 1968. *J. Clin. Invest.* 47:466
35. Garg, S. K., Amorosi, E. L., Karpatkin, S. 1971. *N. Engl. J. Med.* 284:11–17
36. Harker, L. A., Slichter, S. J. 1972. *N. Engl. J. Med.* 287:155–59
37. Harker, L. A. Unpublished observation
38. Evatt, B. L., Levin, J. 1969. *J. Clin. Invest.* 48:1615
39. Najean, Y., Ardaillou, N. 1969. *Scand. J. Haematol.* 6:395
40. Harker, L. A. 1970. *Am. J. Physiol.* 218:1376
41. Odell, T. T. Jr., Jackson, C. W., Friday, T. J. 1970. *Blood* 35:775
42. Harker, L. A. 1970. *Brit. J. Haematol.* 19:95
43. Kinet-Denoel, C., Bassleer, R., Andrien, J. M., Paulus, J. M. 1971. *Platelet Kinetics*, ed. J. M. Paulus, p. 280. Amsterdam: North-Holland
44. Slichter, S. J., Funk, D. C., Leandoer, L. E., Harker, L. A. 1974. *Brit. J. Haematol.* In press
45. Shreiner, D. P., Levin, J. 1970. *J. Clin. Invest.* 49:1709
46. Odell, T. T. Jr., Tausche, F. G., Gude, W. D. 1955. *Am. J. Physiol.* 180:491–94
47. Odell, T. T. Jr., McDonald, T. P. 1964. *Am. J. Physiol.* 206:580–84
48. Kelemen, E., Cserhati, I., Tanos, B. 1958. *Acta Haematol.* 20:350–55
49. Odell, T. T. Jr., McDonald, T. P., Detwiler, T. C. 1961. *Proc. Soc. Exp. Biol. Med.* 108:428–31
50. Spector, B. 1961. *Proc. Soc. Exp. Biol. Med.* 108:146–49
51. Le-Xuan-Chat, Ebbe, S. 1962. *Med. Exp.* 7:317–23
52. Linman, J. W., Pierre, R. V. 1962. *Proc. Soc. Exp. Biol. Med.* 110:463
53. Rak, K. 1963. *Acta Med. Acad. Sci. Hung.* 19:67–75
54. Buonanno, G., Coraggio, F., Catalano, G., Cocco, F., Formisano, S. 1965. *Minerva Med.* 56:4398–402
55. Schulman, I., Abildgaard, C. F., Cornet, J. A., Simone, J. V., Currimbhoy, Z. 1965. *J. Pediat.* 66:604–12
56. Steinberg, B., Cheng, F. 1965. *Acta Haematol.* 33:86
57. deGabriele, C., Pennington, D. C. 1967. *Brit. J. Haematol.* 13:210
58. Abildgaard, C. F., Simone, J. V. 1967. *Semin. Hematol.* 4:424
59. Choi, S. I., McClure, P. D., Vranic, M. 1968. *Brit. J. Haematol.* 15:345
60. Odell, T. T. Jr., McDonald, T. P., Howsden, F. 1964. *J. Lab. Clin. Med.* 64:418–24
61. Penington, D. G. 1969. *Brit. Med. J.* 4:782–84
62. Cooper, G. W., Cooper, B., Chang, C. 1970. *Proc. Soc. Exp. Biol. Med.* 134:1123–27
63. Penington, D. G. 1970. *Brit. Med. J.* 1:606–8
64. Evatt, B. L., Levin, J. Shreiner, D. P. 1972. *Blood* 40:591–92
65. Nakeff, A., Dicke, K. A. 1972. *Exp. Hematol.* 22:58
66. McDonald, T. P. 1973. *Blood* 41:219
67. Harker, L. A. 1971. *Clin. Res.* 19:179
68. Paulus, J. M., Ed. 1971. *Platelet Kinetics.* Amsterdam: Elsevier. Vol. 2. 360 pp.
69. deGabriele, G., Penington, D. G. 1967. *Brit. J. Haematol.* 13:384
70. Aster, R. H. 1967. *J. Lab. Clin. Med.* 70:736
71. von Behrens, W. E. 1972. *Thromb. Diath. Haemorrh.* 27:159–72
72. Harker, L. A. 1971. *J. Lab. Clin. Med.* 77:247
73. Paulus, J. M. 1967. *Blood* 29:407
74. Keleman, E., Rak, K., Cserhati, I., Lehoczky, D. 1961. *Acta Med. Hung.* 17:293
75. Doan, C. A. 1949. *Bull. NY Acad. Med.* 25:625
76. Dameshek, W. 1955. *Bull NY Acad. Med.* 31:113
77. Moolten, S. E. 1953. *NY J. Med.* 53:550
78. Cooney, O. P., Blatt, W. F., Louis-Ferdinand, R., Smith, B. 1965. *Scand. J. Haematol.* 2:195
79. Brandt, P. W. T., Dacie, J. V., Steiner, R. E., Szur, L. 1963. *Brit. Med. J.* 1:468
80. Siri, W. E. et al 1966. *J. Appl. Physiol.* 21:73
81. Hirsch, J., Dacie, J. V. 1966. *Brit. J. Haematol.* 12:44
82. Ebbe, S. 1968. *Ser. Haematol.* 1:65

83. Gross, S., Keefer, V., Liebman, J. 1968. *Pediatrics* 42:651
84. Shreiner, D. P., Levin, J. 1971. *14th Ann. Amer. Soc. Hematol. Meet. Prog.* Abstr. 351
85. Pohle, F. J. 1939. *Am. J. Med.* 197:40
86. Shaper, A. B., Kear, J., MacIntosh, D. M., Kyobe, J., Njama, D. 1968. *J. Obstet. Gynaecol. Brit. Commonw.* 75: 433
87. Tyslowitz, R., Dingemans, E. 1941. *Endocrinology* 29:817
88. Shaper, A. G. 1968. *Lancet* 2:569
89. Cohen, P., Gardner, F. H. 1961. *N. Engl. J. Med.* 265:611

Copyright 1974. All rights reserved

MEDICAL ASPECTS OF RECREATIONAL SKIN AND SCUBA DIVING

◆7121

Jay P. Sanford, M.D.
Department of Internal Medicine, University of Texas Southwestern Medical School, Dallas, Texas

Skin and scuba diving are becoming increasingly popular, with at least eight million individuals in the United States participating not only along the seacoasts but also in lakes and rivers throughout the country. Hence, an understanding of the physiological principles and medical problems that can arise in the course of such activities should be familiar to most physicians.

EQUIPMENT

The skin diver uses only fins, mask, and usually a snorkel. His diving depth and duration are limited to a single breath. Scuba (self-contained underwater breathing apparatus) provides for a supply of air (or oxygen or other gases) and permits extension of both diving depth and duration. The usual recreational scuba is designed as open circuit, i.e. there is no rebreathing system, so approximately three fourths of each breath is exhausted. While wasteful of gas, such equipment is simpler in design and safer than semiclosed or closed scuba, which requires the incorporation of a carbon dioxide absorber. This review is limited to consideration of skin diving and open circuit scuba diving. There are physiological considerations and medical problems common to both forms of diving, but there are also dissimilarities.

PHYSICAL PRINCIPLES

Consideration of the medical problems that may complicate either skin or scuba diving is facilitated by a review of some of the physical properties of water and air, and the influence of these characteristics on the physiological responses of man. These physical properties include pressure, buoyancy, temperature, refractive properties, conduction of sound, and the behavior of gases.

Pressure

Standard atmospheric pressure is 760 mm Hg [14.7 lb/sq in (psi) or 1033.3 g/cm^2] at 0°C. This is approximately the pressure to which man is subjected at sea level prior to entering the water, and is defined as one atmosphere absolute. In most underwater operations, depth pressure gauges give a zero reading at the surface. To avoid confusion, the word gauge should be included whenever pressures are not absolute, e.g., 2 atm absolute is 1520 mm Hg, or 29.4 psi, or 1 atm gauge, or 14.7 psig. The density of salt water is 64 lb/ft^3, while that of fresh water is 62 lb/ft^3. Each 33 ft of depth from the surface in salt water exerts an additional 1 atm or 14.7 psi. The pressure at any level equals depth in feet times 0.445 psig. The surface area of an average size man is 19.4 ft^2; at sea level the body is subjected to 14.7 psi or 41,000 lb, while at a depth of 33 feet the body is subjected to 82,000 lb. As the human body composition is approximately 65% fluid, it follows many of the laws that apply to fluids. If pressure is applied to the surface of a fluid, it is transmitted equally to all parts of that fluid, and the pressure at any point in a fluid is the same in all directions if the fluid is at rest. Thus, even at great depths vascular pulses and muscular organs function normally despite the environmental pressure. Pressure changes are of significance only when pressure gradients exist.

Buoyancy

According to Archimedes' Principle, an object immersed partially or wholly in liquid is buoyed up by a force equal to the weight of the liquid displaced. Displacement of 1 ft^3 of seawater creates an upward force of 64 lb. A comparison of the density of an object with that of water determines its buoyancy. The density of man at residual lung volume varies between 1.021 g/cm^3 for the very obese to 1.097 g/cm^3 for the lean individual. The density of salt water is 1.027 g/cm^3, while that of fresh water is 1.000 g/cm^3. Thus, at residual lung volume everyone will tend to sink in fresh water, while the individual with a great deal of adipose tissue will float in salt water. The technique used for floating consists of inspiration to increase chest volume, resulting in a greater displacement of fluid with essentially no increase in weight (decrease in density), hence greater buoyancy. In skin diving the pressure will compress the chest and may be sufficient to compress it to residual volume, i.e. at 5 atm or 132 ft the chest volume of an individual which was 6 l would be reduced to 1.2 l with resultant increase in density (negative buoyancy).

Temperature

In most circumstances, diving is done in water cooler than skin, and hence chilling is a problem, especially since water has much greater heat capacity than air and conducts heat away from the body better than air. The normal response to increased heat loss is peripheral vasoconstriction and increased heat production through shivering. This response will permit an individual to spend about 1 hr comfortably in water of 78–85°F (1). Navy studies have shown maximal tolerance of up to 3 hr for divers in only bathing suits in 70°F water (2). In 60°F water this is decreased to about 1½ hr, and in water below this temperature protective suits must be worn for comfortable and safe diving.

Refractive Properties

Normally, most of the refraction of light that focuses the image on the retina occurs at the corneal-air interface. When water is in direct contact with the anterior surface of the cornea, which has almost the same refractive index as water, there is loss of approximately two thirds of the refracting power, so the diver becomes farsighted with 50 diopter hyperopia (3, 4). Under most ideal circumstances, an underwater object has to be at least ten times as large as a similar object in air to be seen (3). If eyes are to be of any real use underwater, they must be protected with goggles or mask, with an intervening air space. However, the use of a mask brings with it a second interface at which the angle of divergence of light passing from water to air is further increased by refraction so that objects appear to be about three quarters of their actual distance away, and about 30% larger (4).

Conduction of Sound

In water, sound travels at 4700 ft/sec and can travel great distances. The increased speed of sound in water makes it virtually impossible for man to localize the direction of the source of a sound.

Behavior of Gases

In considering man's reaction to pressure, the effect of the environmental pressure on the air contained in organs such as the lungs, middle ears, sinuses, and viscera must be considered. The incompressible tissue, muscle, skeleton, blood, etc are undistorted. However, if the pressure in the enclosed air cannot be equalized by supplying compressed air at a pressure equal to the ambient water pressure, either the surrounding tissues must contract or a pressure differential will develop with pathological consequences. Since air and other gases obey the gas laws, an understanding of some of these laws facilitate the understanding of underwater physiology.

BOYLE'S LAW "The pressure of a given quantity of gas whose temperature remains unchanged varies inversely as its volume." This means that if the pressure of a given amount of gas is doubled, it must be compressed to one half its volume ($V_2 = P_1 V_1/P_2$). [Example: if a diver's chest volume is 6 l at sea level (1 atm), at a depth of 33 ft (2 atm) it will be 3 l, or at a depth of 132 ft (5 atm) it will be 1.2 l, which is residual volume (5, 6).] Usual scuba tanks hold air under 2150 psig pressure in a volume of 0.5 ft^3; this amounts to 72 ft^3 of air when at 1 atm. With moderate exercise a swimmer exhales approximately 1 ft^3 of air (referred to 1 atm) per min; thus, at the surface a tank would last 72 min (7). However, at 33 ft, the discharged volume also is one half that at the surface, so that same tank would last only 36 min. Conversely, if the pressure of a given amount of gas is reduced by one half, its volume will double (hence, if a diver was using scuba and had a chest volume of 6 l at 33 ft and during ascent held his breath, the volume would expand to 12 l with the potentially disastrous consequences of air embolism).

DALTON'S LAW "In a mixture of gases the pressure exerted by one of those gases is the same as it would exert if it alone occupied the same volume." Dry air contains

79.03% nitrogen, 20.92% oxygen, 0.04% carbon dioxide, and traces of other rare gases and carbon monoxide. Fortunately, the ratio of CO to O_2 is not changed; otherwise significant increments of carboxyhemoglobin might occur. At sea level this mixture exerts a pressure of 760 mm Hg, i.e. nitrogen 79% of 760 = 600 mm Hg; oxygen 20.9% of 760 = 159 mm Hg. At a depth of 33 ft, pO_2 = 1520 mm Hg X 20.9% = 318 mm Hg. At 4.78 atm (124 ft), the pO_2 of air is 760 mm Hg or the equivalent of breathing 100% oxygen at the surface. At depths of approximately 300 ft oxygen toxicity may become a problem and special gas mixtures rather than air must be used.

HENRY'S LAW "At a constant temperature, the amount of a gas which dissolves in a liquid, with which it is in contact, is proportional to the partial pressure of that gas." The actual volume dissolved depends not only on the partial pressure but also on the temperature and coefficient of solubility of the gas. If pure nitrogen at 1 atm is in contact with water at 37.5°C, equilibrium is reached when the water contains 1.22 ml of dissolved nitrogen/100 ml of water (8). If water is exposed to a nitrogen partial pressure of 600 mm Hg, then 0.96 ml N_2/100 ml of water is dissolved; and if exposed to a nitrogen pressure of 5 atm (132 ft), 5.44 ml of N_2/100 ml would be dissolved. When air is breathed at increased pressure, blood N_2 rises, followed by an increase in tissue uptake. If tissue nitrogen tension becomes high enough, nitrogen narcosis can ensue and after the tissue stores of nitrogen have become increased, rapid decompression can cause dissolution of nitrogen in tissues and blood causing the "bends."

PHYSIOLOGICAL CONSIDERATIONS

Mechanics of Respiration

During inspiration as the chest wall expands, the intrapleural pressure falls with the movement of air into the lungs. With maximal inspiratory effort, the maximal pressure differential is 50–125 mm Hg below atmospheric. This means that the maximal possible length for a snorkel is about 4.5 ft (100 mm Hg) (9). With diving equipment the dead space is increased since the air in the tubes of the scuba contributes to the physiologic dead space. Gas density and nonelastic resistance to airflow increases. With the increase in dead space, either the rate of respiration or tidal volume must be increased to maintain alveolar ventilation. Both the increased ventilation and the increased nonelastic resistance will increase the work of breathing. The exhausted diver typically takes rapid shallow breaths which can seriously reduce alveolar ventilation.

Oxygen and Carbon Dioxide Transport

Oxygen is carried in the blood in two forms: physically dissolved in plasma and combined with hemoglobin. At 1 atm, the plasma solubility of oxygen is low; however, according to Henry's Law the volume dissolved will increase proportionately with the partial pressure of oxygen. This becomes important in carbon dioxide transport. Most CO_2 is hydrated to form carbonic acid which dissociates to H^+ and HCO_3^-. The major buffer for the H^+ is hemoglobin and this buffer action is enhanced by loss of oxygen. In the lungs the hemoglobin takes up O_2 and releases CO_2 from carbonic acid; hence the reactions reverse from those in tissues. Under high pressure, plasma-dissolved O_2 supplies a larger proportion of tissue needs. O_2 is not released from hemoglobin, which consequently lowers the amount of CO_2 transported (Haldane effect) for a given rise in pCO_2. However, this rise in pCO_2 would not be sufficient to cause CO_2 retention nor would it cause mental depression.

Respiratory Stimulation

The stimulus to breathe with breath holding occurs as a consequence of the combined effect of a rising pCO_2 and a falling pO_2. The average arterial pCO_2 at the breaking point of breath holding is 60 mm Hg at a pO_2 of about 80 mm Hg, but this will vary with oxygen tension. A higher pCO_2 is allowed when the oxygen tension is high (i.e. 70 mm Hg at a $pO_2 > 300$ mm Hg), but at a PaO_2 of 50 mm Hg, $PaCO_2$ will not be allowed to rise above about 50 mm Hg before breath holding breaks (10). At PaO_2 of 30 mm Hg, a $PaCO_2$ of only 40 mm Hg will terminate breath holding. Hyperventilation before skin diving is a common practice but is potentially dangerous. While hyperventilation will increase breath holding time, the pCO_2 may be reduced to as low as 15 mm Hg, and the pO_2 increased to 140 mm Hg, the blood content of oxygen will not increase proportionately (11). Under these circumstances during breath holding, the pCO_2 may not increase rapidly enough to end the apnea before loss of consciousness from a decreased pO_2 (12). This occurs because the capacity of the body to store CO_2 is large in comparison with its capacity to store O_2. If CO_2 stores are depleted from the tissue by prolonged hyperventilation, these stores must be repleted as the $PaCO_2$ rises and this prolongs the time required for the $PaCO_2$ to increase to a point at which it will break breath holding. The capacity for O_2 storage will not be affected significantly by hyperventilation and pO_2 will decrease at a rate almost independent of previous hyperventilation (Figure 1). This problem is most severe when diving to depths causing significant pressure increases. The pressure raises O_2 tension, which prolongs breath holding and clear mentation; when the diver feels the necessity to breathe, he starts to the surface, both pO_2 and pCO_2 fall rapidly, and pO_2 may reach levels causing unconsciousness.

MEDICAL EVALUATION FOR DIVING

All persons considering diving should have a complete physical examination including a chest radiograph. General factors to be considered include capacity for enduring physical stress, emotional ability to deal with frightening situations, and the ability to equalize pressure in the ears and sinuses. In addition to the general evaluation of fitness, special attention must be directed toward the presence of pulmonary lesions which might result in air trapping, e.g. asthma, emphysema, previous tuberculosis, blebs, cystic disease, or other cavitary lesions. Any of these predispose to air embolism and should be absolute contraindications to diving (13, 14). Other conditions which may preclude diving include coronary artery disease or convulsive disorders, since an attack while submerged is likely to be lethal.

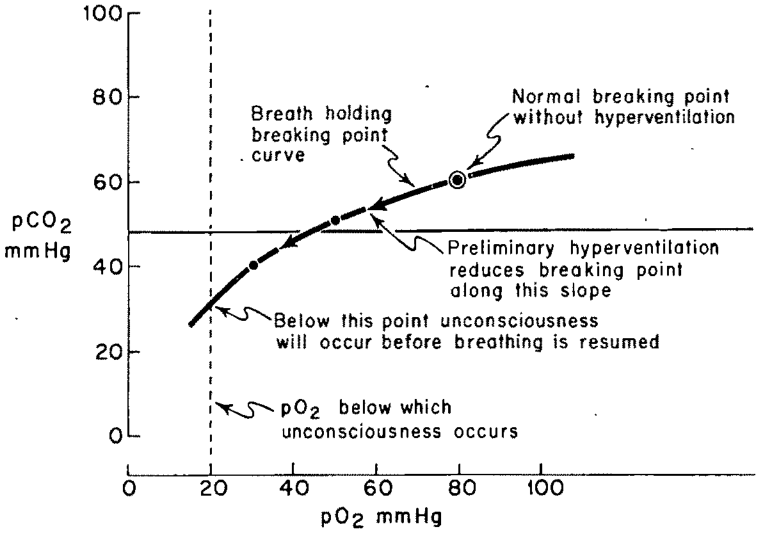


Figure 1 Breath holding "breaking point" curve.

COMPLICATIONS

Most underwater accidents could be prevented by application of the principles which underlie sound diving, by good judgment on the part of the diver, and by sound medical advice. Miles has summarized a series of 194 diving accidents, 51 of which were fatal (Table 1) (15). Many of these causes would be grouped together as drowning, which in the United States is the most common cause of death in skin and scuba diving. The potential medical problems differ between skin and scuba diving (Table 2) (16).

Asphyxia

Asphyxia usually occurs when the air supply is inadequate or the breathing apparatus fails. Most failures can be attributed to carelessness.

Barotrauma (Squeeze)

Pressure differentials across air spaces can result in discomfort and injury. If the air space is noncollapsible, e.g. sinuses, middle ear, and external ear canal with ear plugs, and unless air under high pressure can enter the space, a relative negative pressure gradient will develop. With such a relative negative pressure gradient, the earliest signs are edema and capillary dilatation, followed by capillary rupture and hemorrhage. With scuba the pressure of the inspired air is the same as the surrounding pressure. Thus, intrathoracic pressure is equal to sea pressure and barotrauma only occurs when equalization is precluded by mechanical or anatomic defects.

When eustachian tube blockage prevents equalization, barotrauma results in aerotitis with hemorrhage into the middle ear with or without perforation of the tympanic membrane. With perforation, the concomitant inrush of cold water may result in serious vertigo. Prevention depends upon the ability to equalize pressure by swallowing, yawning, or performing a Valsalva maneuver. Blockage of the sinus ostia results in similar changes, aerosinusitis. If the diver fails to exhale into the face mask during descent, face mask squeeze may occur with conjunctival hemorrhages and facial ecchymosis. Ear plugs are contraindicated in diving because they may result

Table 1 Nature of diving accidents

Cause	Number
Syncope and collapse	46
Decompression sickness	35
Asphyxia	33
Anoxia	24
Pulmonary barotrauma	13
Oxygen poisoning	10
Ears and vertigo	10
Illness in water	9
Coronary thrombosis (3)	
Acute pneumonia (3)	
Epilepsy (3)	
Shark bite	3
Other causes	11
Total	194

Table 2 Medical problems associated with diving

Problem	Type of Diving	
	Skin	Scuba
Drowning	Yes	Yes
Barotrauma	Yes	Yes
Air embolism	No	Yes
Decompression illness	Unlikely	Yes
Oxygen poisoning	No	Unlikely
Nitrogen narcosis	Unlikely	Yes
Carbon monoxide poisoning	No	Yes

in external ear squeeze. Gas in the gastrointestinal tract causes little difficulty because of distensibility of the intestine.

If a diver breathes normally during descent, the air that expands is exhaled normally; in contrast, if he holds his breath or has localized airway obstruction, the entrapped air expands and may result in tears in the alveolar membrane with development of mediastinal and subcutaneous emphysema or tears into pulmonary vessels with the occurrence of air emboli. Tissue rupture can occur with transpulmonic pressure differentials of as little as 60 mm Hg, so that breath holding while rising as little as 10 ft may be dangerous (17). Air emboli lodge in smaller arterioles to produce infarction. In a group of patients with pulmonary barotrauma, Miles & Wright noted the following clinical findings: unconsciousness, 16; pain in the chest, 13; weakness and paralysis, 13; disorientation, 9; cough, 6; visual impairment, 4; convulsions, 3; breathlessness, 2; and cyanosis, 2 (18). Treatment consists of immediate recompression to cause the emboli to shrink in volume.

Decompression Illness (Bends)

Decompression illness and cerebral embolization are both caused by gas bubbles; however, the gas in decompression illness is nitrogen in the form of bubbles in systemic veins and tissues, in contrast to air embolism which is due to air in lodged arterioles (5). During rapid decompression the amount of gas leaving the tissues may exceed that which can be dissolved in blood with the formation of bubbles. A 50% decrease in ambient pressure can occur safely, i.e. an individual may ascend from 6 atm to 3 atm or 2 atm to 1 atm, but not directly from 3 atm to 1 atm (19). In dives of less than 33 ft, gradual decompression is not required since the maximum gas pressure cannot exceed 2 atm.

Scuba divers should stay within "no decompression" limits; however, there are aspects to keep in mind. The duration of time, not depth, determines the degree of saturation of blood with nitrogen, while the total quantity of nitrogen absorbed is determined by the depth of the dive. Depth refers to the deepest point regardless of time spent at the point. For the sport diver, repetitive dives are important since complete desaturation does not transpire for up to 12 hr. Multiple dives within a 12 hr period continue to add to gas tension. An interval of less than 10 min is treated as no break between dives. Thus, repeated no compression dives can result in the bends. Scuba diving before flying in unpressurized aircraft is even more hazardous. Individuals should not be allowed to fly above 18,000 ft within 12 hr of diving below 30 ft (20).

Clinical features of decompression illness occur early, and any vague or unusual symptom should be regarded as decompression illness (Table 3) (21). Within the first hour, 50–85% of individuals will develop symptoms, with 90–97% by 12 hr (21). Neurologic findings occur earliest and include involvement of the brain and spinal cord as well as peripheral nerves, with symptoms relating to spinal cord involvement being more common (22). Symptoms of spinal cord lesions include paralysis, altered reflexes, and sensory changes. The most common neurologic residual is incontinence of urine and feces. The most common symptom is pain in the arms or legs, especially about the joints, and in 66–95%, it is the only symptom (21). Only 5–8% of patients

Table 3 Symptoms and signs in decompression illness (935 cases)

Symptom or Sign	Frequency (%)
Localized pain	91.8
Numbness or paresthesia	21.2
Muscular weakness	20.6
Skin rash	14.9
Dizziness or vertigo	8.5
Nausea or vomiting	7.9
Visual disturbances	6.8
Paralysis	6.1
Headache	3.9
Unconsciousness	2.7
Urinary disturbances	2.5
Dyspnea (chokes)	2.0

with bends have no limb pains. Other clinical features include skin involvement consisting of early localized burning or tingling. Prominent areas involved include the pectoral region, back or shoulders, upper abdomen, forearms, and thighs. These areas have increased fat deposits and may remain tender for days. Pulmonary symptoms (chokes) include acute dyspnea, substernal pain, and paroxysmal coughing which may precede collapse and shock. Individuals susceptible to diver's mottled skin rash seem to have an increased incidence of chokes (18). Aseptic necrosis in bones also may occur (23). Treatment consists of rapid recompression.

Poisoning by Gas

OXYGEN POISONING For the recreational diver with open circuit scuba, oxygen poisoning should not be a problem since it usually requires dives in excess of 300 ft. With 100% O_2, the limit should be 120 min at 33 ft, not to exceed 60 ft (24). Clinical symptoms of acute oxygen poisoning include lip twitching, dizziness, nausea, choking sensation, dyspnea, tremor, and convulsions (25).

NITROGEN NARCOSIS Nitrogen pressure must be increased before nitrogen becomes narcotic. The possibility of nitrogen narcosis limits the effective depth for an air-supplied diver to less than 250 ft and nitrogen narcosis has been reported to occur at 30 ft (26). The clinical features most often develop after descent to 100–150 ft and consist of lightheadedness, increase in self-confidence, loss of fine discrimination, and some euphoria (27). At 150–200 ft, garrulousness occurs. By 200–250 ft, the symptoms progress to include uncontrolled laughter approaching hysteria, decreased ability to concentrate, peripheral numbness, and less attention to personal safety. At 300 ft there is depression and loss of ability to think clearly with unconsciousness usually occurring by 350 ft. Predisposing factors include antecedent fatigue and recent ingestion of alcohol. Prevention is best accomplished by limiting the depth for inexperienced divers to 150 ft and to 250 ft for the experienced. Treatment consists of allowing time for gas elimination. Narcosis at 250 ft may not disappear until the individual reaches 50 ft.

CARBON MONOXIDE POISONING A number of contaminants may be encountered in compressed air, carbon monoxide being an important one. The source of carbon monoxide is usually either from the partial combustion of the lubricant in oil-lubricated high pressure compressors where cylinder temperatures may become high enough to produce partial combustion or from filling scuba tanks near the exhaust from the compressor. Air in scuba tanks should contain less than 20 ppm of carbon monoxide (28).

ACKNOWLEDGMENT

I wish to express my appreciation to Robert L. Johnson Jr., M.D. for his critical review and helpful suggestions.

Literature Cited

1. Dueker, C. W. 1970. *Medical Aspects of Sport Diving,* p. 48. Cranbury, NJ: Barnes
2. U.S. Navy Department. 1963. *U.S. Navy Diving Manual.* Washington, DC: GPO
3. Barnard, E. E. P. 1961. *Proc. Roy. Soc. Med.* 54:755–56
4. Kinney, J. S., Luria, S. M. 1971. *Vision and Visibility in Underwater Physiology,* ed. C. J. Lambertson, 271–76. New York: Academic
5. Miles, S. 1969. In *Underwater Medicine,* p. 165. Philadelphia: Lippincott
6. Carey, C. R., Schaefer, K. E., Alvis, H. J. 1956. *J. Appl. Physiol.* 8:519–23
7. Dewey, A. W. Jr. 1962. *N. Engl. J. Med.* 267:759–65, 812–20
8. Miles, S. See Ref. 5, p. 35
9. Dittmer, D. S., Grebe, R. M., Eds. 1958. In *Handbook of Respiration,* p. 134. Philadelphia: Saunders
10. Mithoefer, J. C. 1965. *Handb. Physiol. Sect. 3* 7:1011–27
11. Dimitru, A. P., Hamilton, F. G. 1964. *Gen. Pract.* 29:123–25
12. Craig, A. B. Jr. 1962. *J. Sport Med.* 2:23–26
13. Davies, W. 1961. *Practitioner* 187: 783–86
14. Williamson, D. E. 1965. *J. Fl. Med. Assoc.* 52:712–13
15. Miles, S. See Ref. 5, p. 217
16. Dueker, C. W. See Ref. 1, p. 36
17. Shaefer, K. E., McNulty, W. P. Jr., Carey, C., Liebow, A. A. 1958. *J. Appl. Physiol.* 13:15–29
18. Miles, S. See Ref. 5, p. 86
19. Dueker, C. W. See Ref. 1, p. 148
20. Furry, D. E., Reeves, E., Beckman, E. 1967. *Aerosp. Med.* 38:825–28
21. Rivera, J. C. 1964. *Mil. Med.* 129:314–34
22. Merrill, R. H. 1970. *Mil. Med.* 135:464–70
23. James, C. C. M. 1945. *Lancet* 2:6–8
24. Lambertsen, C. J. 1947. *Occup. Med.* 3:230–45
25. Donald, K. W. 1947. *Brit. Med. J.* 1:667–72, 712–17
26. Dueker, C. W. See Ref. 1, p. 113
27. Miles, S. See Ref. 6, p. 112
28. Breysse, P. A. 1965. *Ind. Med. Surg.* 34:870–73

Copyright 1974. All rights reserved

THE EFFECTS OF FLUORINATED ANESTHETICS ON THE LIVER AND KIDNEYS

❖7122

Leonard S. Gottlieb, M.D., M.P.H. and Charles Trey, M.D., M.B.Ch.B.
Mallory Institute of Pathology and Thorndike Memorial Laboratory, Boston City Hospital; Departments of Pathology, Boston University School of Medicine and Harvard Medical School; Departments of Gastroenterology, New England Deaconess Hospital and New England Baptist Hospital; and Department of Medicine, Harvard Medical School

Halothane and methoxyflurane are the most commonly used fluorinated anesthetic agents and have a relatively short history. Halothane was planned and developed in the late 1950s as an agent suitable for inhalation anesthesia. It is a liquid with a low boiling point and therefore a vapor pressure facilitating administration. It is nonflammable and has a reasonably high anesthetic action at low concentration with a good safety margin. After extensive investigation, halothane was thought not to cause damage to vital organs. In many hospitals, halothane is used in more than 70% of all cases requiring general anesthesia; in some hospitals in England the figure approaches 90% (1–8).

EFFECTS OF FLUORINATED ANESTHETICS ON THE LIVER

The first clinical trials were conducted by Johnstone (9) in 1956; since then the pharmacology of halothane has been extensively studied and reviewed. The first reports of postoperative hepatitis possibly related to the administration of halothane began to appear in 1958 (10, 11), the year it was introduced into clinical use in the United States. Subsequent reports of fatal cases led to the National Halothane Study, a retrospective study which assessed the possible association of fatal postoperative massive hepatic necrosis with the administration of halothane and other anesthetic agents (12). Controversy still rages over the effect of halothane on the liver and the incidence of the clinical syndromes of postoperative hepatitis and fulminant hepatic necrosis associated with halothane exposure (13–23). The Symposium on Anaesthesia and the Liver (24) and the Danish Symposium in 1972 on Biochemical Effects of Halothane vividly illustrated this controversy (25). In January, 1973, there were two additional symposia on this subject, one from England (26) and the other from Canada (27). In three of the four symposia there were contentions by the same anesthesiologists (5) that there is no relationship between the administration of halothane and the onset of hepatic necrosis and, indeed, that no such syndrome exists; internists (20, 28, 29) asserted that halothane-associated hepatic necrosis is a definite entity. As in earlier reports in an American journal in 1969, the internist (30) and anesthesiologist (31) served on the same hospital staff. Carney & Van Dyke, in an extensive review (14), concluded that halothane hepatitis is a real but rare entity. These anesthesiologists suggest that the prudent use of halothane would significantly reduce the incidence of the syndrome of posthalothane hepatitis.

Biotransformation of Halothane and its Effects on the Liver

Until quite recently, halothane was considered biochemically inert. However, in animal experiments and later in man, it was conclusively shown that halothane is extensively metabolized and that the degree of metabolism is greater in man than it is in other species (32). The metabolism of halothane in humans has been studied by quantitating in urine previously labeled fluoride and bromide ions within the halothane molecule (33). Halothane is oxidized to trifluoroacetic acid, inorganic bromide, and chloride. Unlike methoxyflurane, these observations show that the fluoride ion from halothane is not removed, and that the fluoride is excreted as a trifluoroacetic acid. This may be important in explaining the difference in the renal effects caused by halothane and methoxyflurane (34). From 12–25% of halothane is metabolized and the biotransformation occurs slowly with a half-life of 13 days. Van Dyke & Chenoweth found that rats metabolized only 2–4% of a given dose in the same period. They also suggest that there may be transient intermediates which, as yet, have not been identified (35). Brown found that halothane, methoxyflurane, and acetone induce the enzymes for analine hydroxylation (36).

The effects of halothane on isolated perfused rat livers have been studied by Biebuyck et al who demonstrated that halothane stimulates the rate of glycolysis and inhibits gluconeogenesis and urea synthesis. They have also shown that halothane inhibits oxygen consumption. When oleate is added to the perfusion medium in concentrations that could occur in the blood in vivo, halothane effects are abolished. They suggested that halothane per se may have a deleterious effect on liver metabolism (37–39).

Smuckler et al (40) have clearly shown that enzyme induction in the liver is enhanced by halothane. This induction could also be stimulated by phenobarbital and other agents used in premedicating the patient for anesthesia. Smuckler et al noted this induction indirectly by the proliferation of smooth endoplasmic reticulum, while others (34, 41) have shown this induction directly by the measurement of the enzyme cytochrome P-450. The activity of this nonspecific enzyme system for metabolism of drugs is dependent on genetic and environmental influences. Cascorbi et al have shown that 90% of halothane metabolism in identical twins is controlled by genetic factors. The genetic makeup of the individual can also determine the degree of halothane metabolism which can be influenced by the environment (3). Van Dyke noted the accumulation of these metabolites in livers with decreased blood flow and concluded that this may be of potential practical importance in the development of hepatotoxicity related to hypoxic episodes (34). Stenger & Johnson (42) have shown that pretreatment with phenobarbital could lead to patchy centrilobular necrosis in rat livers, as well as hepatic functional changes. These experiments tend to point to a direct but mild hepatotoxic effect by halothane, an effect not apparent in man, nor similar to the reported clinical syndrome.

Paronetto et al (43–45), as well as Doniach et al (46, 47), have shown an elevation of mitochondrial antibody in the sera of patients who were presumed to have halothane-associated hepatitis. Following these observations, Cohen et al (48) measured the mitochondrial antibody levels in rats pretreated with phenobarbital and having either single or double exposures to halothane. Rats with a single exposure to halothane did not demonstrate mitochondrial antibodies, while 62.5% of the rats treated twice with the phenobarbital/halothane sequence demonstrated mitochondrial antibodies in the sera when measured at one or two weeks after the second treatment sequence. It is likely that the development of mitochondrial antibody is related to the recurrent liver injury. At present, there is no experimental model which reproduces the complete syndrome of halothane-associated hepatitis as observed in man.

Clinical Syndrome of Halothane-Associated Hepatitis

By 1968, 10 years after the first reported instances of halothane-associated hepatitis, there were at least 400 case reports (49). From our observations of the first 13 patients with halothane hepatitis that we had encountered in Boston by that time, we defined a clinical syndrome (13) which seemed to encompass most of the cases reported by others (Table 1). The recent reports of Williams and others emphasize these aspects (20, 28, 29, 50). Although the syndrome could occur at any age, it is predominantly seen in patients over 40. Females constitute the majority of cases. Instances in children have rarely been reported. In about 80% of cases, the characteristic history is that of multiple exposures to halothane, usually within a period of about 60 days. This multiplicity would be expected in the sensitizing type of hepatocellular disease. It is contrary to the statistical incidence of single to multiple exposure in the general population. In the National Halothane Study, which monitored 856,000 patients receiving general anesthesia, the chance of receiving multiple exposures to an anesthetic agent within two months was only 9% (12). It thus seems that rechallenge is an important factor in the development of this form of hepatitis. In the case reports we have reviewed, other causes of hepatitis seem remote, and most patients were presumed to be free of previous hepatic disease. The time period of 21 days between exposure and onset of hepatitis was arbitrarily taken, as a longer period would be less relevant. There was no relationship between length of exposure to halothane or the operative risk to the onset of the hepatocellular disease. Most of the patients reported had low-risk operations. However, in some reports of patients who had more than one exposure, the second operation was due to presumed obstructive jaundice, when in fact the correct diagnosis could have been cholestasis due to a previous exposure. No obstructions were found, and in many instances the patients died after subsequent reexposure. These observations are in contrast to the National Halothane Study which reported massive hepatic necrosis following predominantly high-risk operations.

Because of the occurrence of hepatitis after multiple halothane exposures, features following earlier exposure were analyzed in an effort to detect the patients at risk. Unexplained fever was noted in about 80% of the patients. Unexplained fever in the postoperative period is common (51), and has to be considered in relationship to its severity, as well as the type and extent of surgery. In many instances, fever occurred after diagnostic curettage, plastic operations, and other operations where postoperative fever is unusual. Excessive nausea and vomiting occurred on occasion, and eosinophilia was noted in some patients. In the cases reported to date, the onset of jaundice developed from 3–9 postoperative days in about 60% of cases, and in another 30%, on the 10th–14th day. In some instances, the hepatitis was anicteric. The liver pathology noted in all cases was that of hepatitis and could easily be distinguished from shock and congestion. Liver function tests could not distinguish this form of hepatitis from other types of hepatitis. The presence of mitochondrial antibodies in the sera of some of these patients and lymphocyte transformation was noted (43–47).

The prognosis of patients developing this syndrome is unknown. Instances of mild hepatitis have been reported. However, Trey et al have noted a mortality of 97% in 76 patients who developed severe hepatic coma (52).

Table 1 Clinical syndrome of halothane-associated hepatitis

Age: Any age, but predominantly over 40 years.

Sex: Both sexes, females predominate.

Incidence: Not established. Presumed mortality 1:10,000 halothane anesthesias or 1:1000 multiple exposures to halothane.

Clinical Features:

1. Multiple exposures, usually within 60 days.
2. Not dose related.
3. Usually reported in patients without prior history of liver disease.
4. Course varies from anicteric hepatitis to massive hepatic necrosis. Can have cholestatic phase.
5. Unexplained postoperative fever is common.
6. Eosinophilia reported.
7. Jaundice and hepatitis – usually 3 to 21 days postexposure.
8. Cross-reactivity with methoxyflurane.

Pathology: Similar to viral or hepatotoxic hepatitis.

Mode of Action: Halothane undergoes biotransformation. No animal model has reproduced complete syndrome. Hepatitis presumed by sensitization.

Pathology of the Liver Associated with Halothane Anesthesia

The entire spectrum of histopathologic changes of halothane-associated hepatitis from the mild forms of hepatitis to the more severe forms of submassive and massive necrosis is essentially indistinguishable from those observed in viral hepatitis. However, Peters et al recently studied 33 cases of fatal hepatic necrosis and 8 nonfatal cases, and stated that they were able to distinguish this lesion from that of viral hepatitis because of the significant amount of collagen deposited within two weeks following the onset of disease (53). They classified hepatic changes into three more or less definitive stages of evolution. The first stage (necrotic stage) included patients who had survived up to eight days. A zonal type of severe necrosis was noted involving the central two thirds or more of each lobule. At two days, the involved liver cells were acidophilic with granular cytoplasm. At the margin of the lesion near the periphery of the lobule, occasional necrotic acidophilic hepatocytes were present among viable liver cells, many of which showed vacuolar changes. Intralobular cholestasis was present in only a few cases. At four days, small numbers of round cells were present in the portal areas, but were not seen in areas of necrosis. The second stage (resorptive stage) occurred in patients who had survived for over a week. Microscopically necrotic liver cells were absorbed leaving behind a residual of lipochrome-filled macrophages and either congested sinusoids or collapsed ischemic centrilobular areas in which there was condensed stroma and often collagen fibers. In the congested areas, necrosis and autolysis were described as being complete with intralobular and portal round cell infiltrates. In the collapsed ischemic type of change, sinusoids and central veins often could not be identified. The peripheral zone of cells often showed evidence of mild regenerative activity and cholestasis. Bile ductules showed hyperplasia and round cells were present in the portal triads. In the third stage (regenerative stage), usually two weeks or more after the onset of jaundice, the necrotic debris in the centrilobular zone had disappeared and the connective tissue framework appeared compressed. Macrophages and a few lymphocytes were still present. The authors noted a characteristic regeneration in the periphery of the lobule. Cholestasis appeared to be marked with intralobular and intraductular bile plugs.

Uzunalimoglu, Yardley & Boitnott reported on the light and electron microscopic changes in three patients with mild halothane hepatitis (54). They reported that the light microscopic findings in individual cases were indistinguishable from those of viral hepatitis. However, they did note that the patchy nature of the hepatocellular injury and inflammation and the presence of relatively large areas of hepatocellular dropout were unusual in mild viral hepatitis. A single case of halothane hepatitis in which transient granulomas occurred in a patient with a fatal outcome was reported by Dordal and associates (55). The granulomas were observed three days after a second exposure to halothane and were not present at the time of death six weeks later. This is obviously a rare phenomenon and has not been reported by other investigators.

Recent electron microscopic studies by Klion et al (56), Keeley et al (57), and the Johns Hopkins group (54) revealed a wide variety of nonspecific changes in the smooth and rough endoplasmic reticulum, mitochondria, lysosomes, Kupffer cells, and other cytoplasmic organelles and inclusions. We conclude that ultrastructural studies alone cannot distinguish between halothane hepatitis and viral hepatitis.

Pathogenesis of Halothane-Associated Hepatitis

Retrospective studies conducted by Mushin et al (58) and Slater et al (59) claimed no instances of halothane-associated hepatitis in 68,883 patients. In 1973 Greene (60) reviewed the risk of hepatitis from anesthetic exposures in patients previously maintained on drugs capable of producing enzyme induction. Two instances of hepatitis developed in 914 halothane anesthetics, while five instances of hepatitis developed in 206 anesthetics other than halothane. The incidence of postoperative fever was less frequent than fever which occurred incidentally in this patient population. These observations suggest that halothane hepatitis is not dose-related or due to the direct effect of the metabolites on the liver. In reviewing possible other causes in case reports, viral etiologies must constantly be considered, and although tests such as radioimmunoassays for hepatitis-B antigen are now commonly used, many instances of postoperative viral hepatitis can occur when these tests are negative (61). The diagnosis of halothane-associated hepatitis in most reported cases was made by the conformity of the presentation to a clinical syndrome as well as by the inability to ascribe any other cause.

The clinical characteristics of the syndrome of halothane hepatitis have led many to consider the possible mechanism of sensitization. Direct hepatotoxicity in animals has been considered, but no similar human instances have been reported. Multiple exposures, reports of postoperative and unexplained fever, eosinophilia, granuloma formation in the liver, and the presence or transient appearance of mitochondrial antibodies suggest a sensitization, rather than a direct hepatotoxic effect. There have been reports of lymphocyte transformation in patients with halothane-associated hepatitis, but these reports have not been duplicated in many instances (7, 44). The following criteria for the diagnosis of sensitization hepatitis appear to apply: (*a*) the syndrome cannot be reproduced in animals; (*b*) the cases are sporadic in occurrence; and (*c*) it is generally not possible pathologically to diagnose most cases of sensitization hepatitis, since they are histologically similar to other forms of hepatitis (62, 63).

The proof of the hypothesis of sensitization must thus rest on the observation of the clinical syndrome, reports of either direct or fortuitous challenge to individuals, and increased instances of operations where multiple exposure is usual. There are well-documented cases of direct challenge. In 1966 Belfrage et al (64) reported an anesthesiologist who suffered from allergy and developed hepatitis. After recovery, he was rechallenged with halothane and a similar hepatitis reoccurred. He has since given up anesthesiology and has not had another occurrence of the disease (65). Klatskin & Kimberg reported recurrent hepatitis in an anesthesiologist and reproduced the event and syndrome by challenge with a nonanesthetic dose of halothane. Liver biopsies confirmed the acute hepatitis (66). Other cases have also been noted by Combs (30). Instances of fortuitous challenge have been reported (15). Patients developed hepatitis, some of them severe; in other instances halothane was readministered to patients of previous presumed halothane hepatitis without untoward effect.

In reviewing experiences of treatments requiring multiple operations under general anesthesia, there were two studies, one in Australia (67, 68) and one in England (69) of patients receiving halothane anesthesia for the treatment of uterine carcinoma. In Hughes and Powell's report (68), 6 of 300 patients receiving multiple exposures to halothane for the treatment of this disease developed severe hepatocellular jaundice, and 2 died. Of the subsequent 300 patients who did not receive halothane as an anesthetic, there was only one case of cholestatic jaundice secondary to chlorpromazine. They reviewed the epidemiology of hepatitis in the Brisbane community and found no increased instance during this period. From Sheffield (69), there were three cases of severe hepatocellular disease out of 57 cases receiving multiple exposures to halothane. Since they have stopped using this form of anesthesia, no further cases were noted. In Brisbane, as in Sheffield, the association seemed to implicate a sensitization by halothane.

The incidence of halothane-associated hepatitis and the associated mortality is unknown. Peters et al (53) reviewed the experience in the Los Angeles County Hospital in the 30 years prior to the advent of halothane anesthesia and did not find instances of this clinical syndrome. In an analysis of 50,000 patient anesthetics in Montreal, 35,000 of which were halothane, an increase of postoperative hepatic complications was noted since the introduction of halothane. It was estimated that there is 1 death from halothane-associated liver necrosis in about 8000 uneventful anesthetics (70). A similar incidence was suggested by the National Halothane Study (12). This retrospective study showed 82 instances of fatal massive necrosis of the liver present in 11,000 autopsies. In 73 of these patients, the hepatic necrosis was due to shock and was of the coagulative type. In 9, the hepatitis could not be explained and 7 of these 9 patients had received halothane; multiple exposures to the anesthetic had occurred in 4 (71, 72). While this study concluded that there is no increased incidence of mortality with halothane, it was recommended that patients with postoperative unexplained fever or jaundice not be reexposed to this agent. The Fulminant Hepatic Failure Surveillance Study reported that in the first 150 patients, 20% had halothane-associated hepatitis (52). Subsequently, in 600 patients in stage IV coma, the incidence of halothane-associated hepatitis was 18%. Thus, although the incidence in the overall population is not known in this study, a considerable number of patients reported with fulminant hepatic failure are presumed to have had halothane-associated hepatitis. The survival in this form of hepatitis is worse than in comparable groups with viral, drug-induced, or other etiologies.

The paradox of the physicans still reporting cases of halothane-associated hepatitis and some anesthesiologists doubting its existence can be explained by the characteristics of this syndrome. The onset of hepatitis and jaundice usually occurs a few days to three weeks after exposure and in operations where liver disease is an unexpected and occasionally a tragic complication. The patient thus presents as a medical problem. The anesthesiologist is generally notified only when the hepatitis is severe or fatal, if then. The search for other causes is then retrospective.

Prospective studies of a sensitizing illness are unrewarding as clinical studies require frequent blood sampling, large populations, and the fortuitous appearance of susceptible subjects. There is no doubt that these individuals exist. No laboratory test is able to identify the patient at risk of developing hepatitis, even after a previous exposure to halothane; at this point we must rely on clinical judgment.

In assessing the importance of the syndrome, one has to consider the overall beneficial effects of halothane and overall improvement in mortality since the introduction of this anesthetic agent, as well as the chance of the fatal hepatic complication. Since multiple exposures predominate before the onset of this event, it is prudent to review the history of patients undergoing anesthesia. The presence of previous unexplained jaundice and unexplained postoperative fever should contraindicate the reexposure to halothane or methoxyflurane. The mitochondrial antibody test is not suitable for screening purposes, and the lymphocyte transformation test is still controversial. At this stage, aside from warnings afforded by liver function tests, there are no tests to detect the patient at risk. In considering halothane's place in clinical practice, there should be both positive indications for the use of halothane as well as contraindications. Its use should be reserved for the case where this anesthetic can be most beneficial.

Methoxyflurane-Associated Hepatitis

Hepatitis has been reported with methoxyflurane usually after repeated exposures (73–78). There have also been cases of halothane exposure and the subsequent exposure to methoxyflurane as the presumed cause of hepatitis (79). Elkington et al (80) reported a patient who had both renal and liver manifestations of methoxyflurane-associated hepatitis. We have seen a patient with a similar event in whom dehydration occurred because of the patient's comatose state. Brenner & Kaplan (73) reported patients with a mild attack of hepatitis occurring on reexposure to methoxyflurane. The patient had allergies, transient eosinophilia, and hepatitis. Joshi & Conn (81) reviewed the literature and found 21 instances of methoxyflurane-associated hepatitis and reported 3 more in their experience. In their review, the clinical syndrome was similar to that of halothane-associated hepatitis. Fatal cases have been reported (74–79).

EFFECTS OF THE NEWER FLUORINATED ANESTHETICS ON THE LIVER AND KIDNEYS

Newer fluorinated anesthetics such as teflurane, fluroxene, hexafluorobenzene, isoflurane, and enflurane are being introduced into clinical anesthesia practice. Isolated reports with massive hepatic necrosis have been noted with fluroxene (82–85). Reynolds et al (82) reported a fatal case of hepatic parenchymal necrosis of the centrilobular type. The patient had been pretreated with phenobarbital and diphenylhydantoin, drugs which may cause enzyme induction. Tucker et al (83) reported a case of hepatorenal toxicity following fluroxene anesthesia in a patient who had been taking depressant drugs. They noted lesions characterized by centrilobular and mid-zonal coagulation necrosis with loss of nuclei and fatty degeneration. They speculated that the lesion could be related to metabolic degradation of the anesthetic agent. The renal lesions reported by Tucker et al are consistent with acute renal tubular degeneration. The patient also had pulmonary edema and small foci of visceral hemorrhage. These latter lesions could be attributed to shock. A case of nonfatal jaundice associated with fluroxene was reported (84). Johnston et al (85) reported fluroxene toxicity in a variety of experimental animals. Cascorbi (3) recently showed that trifluoroethanol, a metabolite of fluroxene, is most likely a toxic agent and that its toxicity can be decreased by suppressing hepatic enzymatic activity. The other newer agents have not yet been associated with any untoward liver and kidney abnormalities. There are differences in biotransformation among the various fluorinated anesthetics and the effects of one cannot be extrapolated to other fluorinated agents (33, 34).

THE CLINICAL AND PATHOLOGICAL SYNDROME OF METHOXYFLURANE RENAL DISEASE

The association of methoxyflurane administration with nephrotoxicity has been documented in retrospective reports (86–92) and prospective studies (93–97). The clinical syndrome and pathological lesions associated with methoxyflurane effects on the kidneys have now been well described and are summarized in Table 2. There is little doubt that the administration of methoxyflurane has a direct effect on the kidneys. This syndrome is fairly common; it is usually dose-related and has a clinical spectrum ranging from benign to severe nephrotoxicity, from short to prolonged duration, and fatalities have been reported. The benign syndrome was well described by Crandell et al in 1966 (86). They reported that 16 of 94 patients who received methoxyflurane in their hospital developed toxic nephropathy with a syndrome similar to that of nephrogenic diabetes insipidus. Urine osmolality was close to that of the serum. The patients were in negative fluid and electrolyte balance. Some patients were noted to have pronounced weight loss. There was an elevation of the serum sodium, serum osmolality, and blood urea nitrogen (BUN). These patients were unable to concentrate urine despite fluid deprivation or administration of vasopressin. These defects suggested that the lesion was of renal tubular origin. In most cases, renal function impairment was transient—10 to 20 days, but in three cases, the BUN remained elevated for about 12 to 29 months. In the same year Pezzi et al (98) noted a similar syndrome in 16% of 123 patients undergoing abdominal surgery with methoxyflurane. Of 180 patients who were subjected to all types of operations, 20 showed this lesion. However, in their series, some patients suffered severe major medical or surgical complications which they acknowledged might have contributed to the observed renal difficulties. They noted that the onset of the polyuric renal failure can occur within a day of surgery, and can last for a variable length of time from a few days to weeks or months, or even years. The incidence and uniqueness of the renal complications of methoxyflurane anesthesia has been reported by Urgena & Gergis (99). They reviewed the records of 1000 of the 1196 patients receiving methoxyflurane over a 6-year period and noted 2 patients who developed severe renal insufficiency but survived, and another 17 who showed inappropriate postoperative diuresis. The records of 1000 patients who were given other general anesthetics were studied. These patients did not exhibit inappropriate diuresis. However, the mortality rate from all causes was comparable in the two groups.

The relation between the dose and the renal lesion has been elegantly examined in prospective studies by Mazze et al (93–95) and Tobey & Clubb (96), and also noted in a prospective study in children by Black & Keilty (100). Mazze et al explored the possibility of renal dysfunction associated with methoxyflurane anesthesia in males scheduled for elective surgery. Twelve patients received methoxyflurane and ten halothane. Standard maintenance levels of methoxyflurane were given in patients receiving this anesthetic, and standard halothane levels were maintained in the patients receiving halothane. The patients receiving halothane had a mild hypernatremia and serum hyperosmolality postoperatively with a prompt return to preoperative renal concentration ability. Two patients had a transient inappropriate antidiuretic hormone response for the first two postoperative days. The methoxyflurane-anesthetized patients, however, had significant increases in serum sodium, urea

Table 2 Clinical syndrome of methoxyflurane nephropathy

Age and Sex: Any.

Incidence: Mild syndromes and morbidity – common. Deaths reported. Incidence not known – may be rare. Associated hepatic necrosis in few patients.

Clinical Features:

1. Can occur with single or multiple exposure.
2. Probably dose related.
3. Nephrogenic diabetes insipidus. Low osmolality urine, polyuria, ±4 l/day, which does not respond to fluid restriction or antidiuretic hormone administration.
4. Dehydration and electrolyte loss in untreated patient.
5. Duration and severity – variable. Few days to years. Patients may need dialysis or renal transplant. Deaths reported.
6. May be associated with hepatic necrosis.
7. Aggravated by obesity, tetracycline, gentamicin, and prolonged administration of the anesthetic.
8. No demonstrable cross-reaction with halothane.

Pathology: Renal interstitial reaction; intratubular calcium oxalate crystal deposition and fibrosis. Occasional glomerular and renal vascular lesions.

Mode of Action: Methoxyflurane undergoes biotransformation. Metabolites, especially inorganic fluoride ion, affect renal tubules. Dose-related response. Tubular calcium oxalate crystal deposition is an aggravating cause. Lesion reproduced in experimental animals.

nitrogen, uric acid, and osmolality. They showed marked weight loss, and return of renal concentrating ability was markedly delayed. Postoperative potassium excretion was greater after methoxyflurane. All 12 methoxyflurane patients thus had some evidence of renal abnormality, and postoperative polyuria and dehydration added to the difficulties of the clinical management of 6. The changes described by Mazze et al indicated that the lesion was of the distal nephron. The dysfunction had been observed with several lots of methoxyflurane (93–95). Tobey & Clubb (96) observed the effects of methoxyflurane and halothane anesthesia over a period of 20 days in healthy young adults undergoing elective surgery. The concentration and length of administration of these agents were less than the group studied by Mazze. Tobey & Clubb (96) also noted important postoperative differences of blood inorganic fluoride between the patients receiving halothane and methoxyflurane. There was also significant correlation between the venous blood concentration of methoxyflurane at the end of the operation and the level of inorganic fluoride in the first postoperative day. Urinary oxalate crystals were seen postoperatively in 90% of the methoxyflurane group, as compared to 43% of the halothane group. This illustrates that metabolism of the methoxyflurane can lead to the formation of inorganic fluoride ion and oxalic acid, both of which are potentially nephrotoxic. In 8 of 20 methoxyflurane-anesthetized patients, there were frequent oxalate crystals in the urine. These two randomized prospective studies confirm the clinical syndromes described by Crandell et al (86). In a recent prospective clinical study, Cousins & Mazze demonstrated a dose-related nephrotoxic response to methoxyflurane (97). They followed 18 patients who received methoxyflurane and 8 control patients who received halothane anesthesia. None of the control patients developed nephrogenic diabetes insipidus, while 10 of the 18 methoxyflurane patients developed this syndrome. They noted a relationship among the dose of methoxyflurane calculated as minimum alveolar concentration per hour, serum peak inorganic fluoride levels, and the renal effects. Subclinical nephrotoxicity was demonstrated with 2.5 to 3 minimum alveolar concentration hours and clinical toxicity was present with dosages greater than 5 minimum alveolar concentration hours. The peak serum inorganic fluoride level was 50–80 μmol/l in the smaller dose, and 90–120 μmol/l in the larger dosage. In the higher dosages of 7 minimum alveolar concentration hours received by 3 patients, the clinical toxicity was more severe, the peak serum inorganic fluoride levels higher, and the postoperative management more difficult. Patients who have suppressed thirst mechanisms or who are unconscious and develop methoxyflurane nephrotoxicity can become grossly dehydrated. We have noted this in a patient with renal and concomitant liver failure and hepatic encephalopathy (13). Reports of subclinical effects of methoxyflurane in children by Black & Keilty (100) showed that effects may be exposure related. They demonstrated significant reductions in body weight and increases in BUN and plasma uric acid in the postoperative period in normal children, as compared to a similar group receiving halothane. Although methoxyflurane had been considered safe as an anesthetic in obstetrics, case reports of nephrotoxicity have been noted in the mother (101) and there has been concern regarding the effects of methoxyflurane on the fetus (102).

Artusio described a fatal case of methoxyflurane renal failure in 1960 (103). In 1964, Paddock (104) described 4 cases of severe renal failure following the administration of methoxyflurane, and 2 were fatal. Mazze & Cousins reported a patient with mild nephrotoxicity following methoxyflurane anesthesia which rapidly increased in severity when gentamicin therapy was commenced (105). They suggest that the nephrotoxicity of these two drugs may be additive. This sequence of events has been reproduced in the rat (106). In 1966 Pezzi et al (98) reported 20 patients with this syndrome and 6 deaths in his series of 120 patients receiving methoxyflurane. Initially, Kuzucu (107) thought that tetracycline aggravated the complications of methoxyflurane renal failure. He reported 5 deaths among 115 patients who had abdominal or thoracic surgery after the administration of methoxyflurane and the postoperative administration of tetracycline antibiotic. Panner et al (108) reported the death of 2 obese patients receiving methoxyflurane anesthesia following abdominal surgery who developed polyuric renal failure within one day after exposure. In both of these cases, there was concurrent hepatocellular damage. In 1970 Frascino et al (109) reported 3 deaths in 11 patients and studied the pathology in 7 of these. More recently, in 1972, Hollenberg et al (110) documented the progression of renal failure in 3 patients after exposure to methoxyflurane; in all of them there was prolonged renal failure from 98 to as many as 700 days. One patient received a renal transplant, one recovered, and one died on the 111th day. These case reports were extensively documented with the pathological changes associated with methoxyflurane. The most disconcerting feature was the lack of recovery after repeated renal dialysis. The factors responsible for this continuation of renal failure were not clear and concomitant tetracycline was not administered although, in one instance, kanamycin was administered in what the authors considered was a nontoxic dosage.

In 1973, Halpren et al (111) reported 2 patients with prolonged renal failure following methoxyflurane administration. Both cases showed the characteristic syndrome. One of the patients recovered after about 6 months of dialysis treatment, and is still alive 18 months later with a high serum creatinine. The other patient died after 14 months of dialysis.

The Histopathology of Renal Lesions of Methoxyflurane Anesthesia

Deposition of calcium oxalate crystals in renal tubules has been reported by Kuzucu (107), Panner et al (108), Frascino et al (109, 112), Hollenberg et al (110), Halpren et al (111), and Aufderheide (113). Frascino et al (109) reported on renal biopsies of 7 patients in whom renal failure followed methoxyflurane anesthesia. Calcium oxalate crystals proved to be a striking feature in all of the biopsy specimens. In this series, the oxalate crystals were found primarily in the proximal convoluted tubules with tubular dilatation, epithelial cell degeneration, necrosis, and occasional giant-cell reaction. The glomeruli were reported as normal. The degree of crystal deposition did not adequately explain the development of renal failure. There was no apparent relationship between the duration of exposure to methoxyflurane and the histologic severity of the lesion. The 3 fatal cases in this group showed approximately the same amount of intratubular crystals as did the biopsy cases. However, there appeared to be greater interstitial inflammation and fibrosis in the fatal cases. Panner et al (108) described 2 patients who developed renal failure characterized by polyuria and progressive azotemia within 24 hr following surgery. In their cases, numerous crystals were present primarily in the distal convoluted tubules, with lesser numbers of crystals in the cortical collecting tubules, thick limbs of the loop of Henle, and the proximal convoluted tubules. Rare crystals were found within medullary tubules. Tubular cells around crystals were generally flat and atrophic. The interstitial connective tissue had increased mononuclear cells. In 1972, Hollenberg et al (110) reported 3 cases of prolonged renal failure lasting from several months to over a year despite the absence of cortical necrosis. All 3 showed similar morphologic characteristics in biopsy, autopsy, and nephrectomy specimens. Some glomeruli revealed an increase in the mesangial matrix with glomerular hypercellularity and ischemic crescents. The interstitial tissues showed moderate to marked edema, foci of fibrosis, and round cell infiltration. Tubular cells revealed degeneration and atrophy with occasional frankly necrotic cells and a few mitotic figures. Hollenberg and associates also emphasized the presence of a vascular lesion in which the small arteries showed subintimal and medial fibrosis and hyalinization. Large amounts of oxalate crystals were present in the tubules, interstitium, and even in the adventitia of blood vessels (Figures 1 and 2). Halpren et al (111) recently reported cases showing severe interstitial fibrosis, chronic inflammation, and inter-

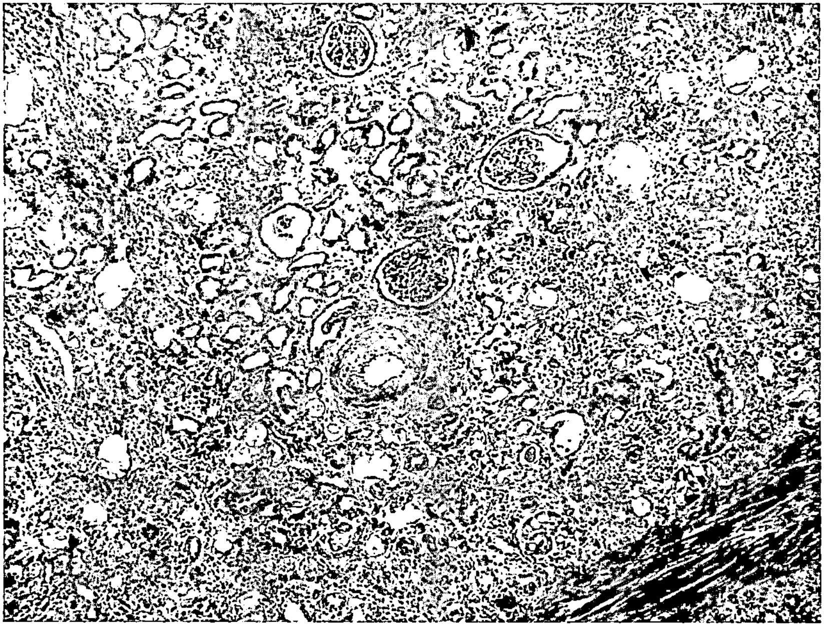


Figure 1 Low power view of kidney showing interstitial fibrosis and round cell infiltrate. Numerous dilated tubules are evident. Oxalate crystals are present in degenerating tubules and in the interstitium. Arterioles and arteries show subintimal fibrosis and hyalinization.

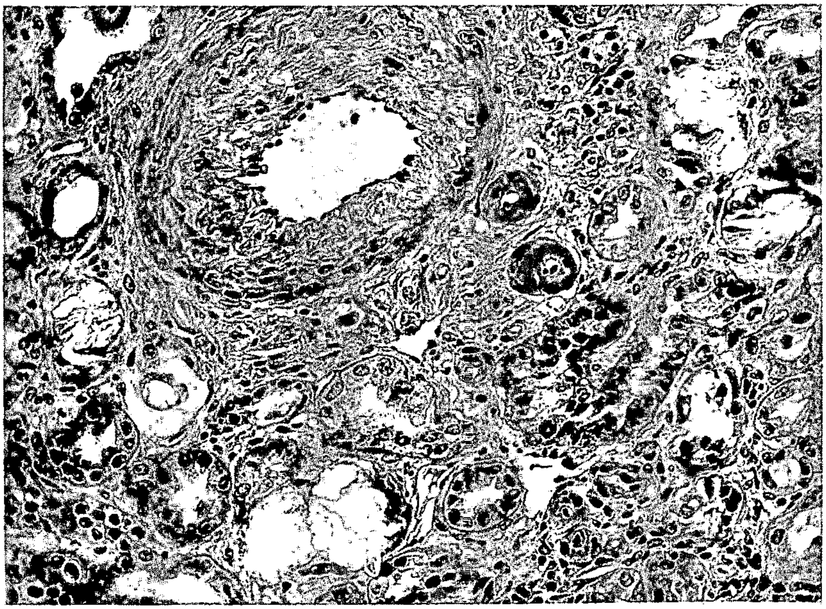


Figure 2 Higher magnification of renal parenchyma near artery shown in Figure 1. This shows (*a*) well-defined oxalate crystals in degenerating tubules and (*b*) the vascular lesions. (Photomicrographs are kindly contributed by Drs. Melvin Schwartz and Ramzi S. Cotran, Mallory Institute of Pathology.)

tubular and interstitial calcium oxalate crystals with extensive tubular loss and atrophy. Electron microscopic studies revealed crystals within the tubules disrupting the epithelium and the basement membrane. In summary, all cases of fatal and nonfatal instances of methoxyflurane renal damage showed deposits of oxalate crystals in the tubules and interstitium with chronic inflammation. To date, only Hollenberg and associates (110) have described a glomerular lesion in their well-studied cases of prolonged acute renal failure

Possible Mechanisms of Action

The possible mechanisms of action of methoxyflurane on the kidney have been studied by Mazze et al (93–95, 97, 105, 106, 115–117), Holaday et al (114), Taves et al (118), and others. Holaday et al (114) labeled methoxyflurane with ^{14}C in the methoxyl position. They showed, in man, that the biodegradation of methoxyflurane began immediately after the onset of exposure and continued to 12 hr. The identified products of the biotransformation were carbon dioxide, inorganic fluoride ion, dichloroacetic acid, and methoxyfluoroacetic acid. They concluded that fluoride ion and oxalate were both nephrotoxic and thus the prime toxicologic suspects. Frascino et al (109, 112) studied the effects of inorganic fluoride on the renal concentration mechanisms in dogs. The high blood fluoride levels interfere with both the generation of maximally concentrated urine and tubular free water reabsorption. Mazze et al (117) showed similar metabolic pathways to that of Holaday. Mazze found greater concentration of inorganic fluoride in patients who received methoxyflurane and developed polyuria than in those who did not develop renal failure. Fluoride inhibits anaerobic metabolism in renal medulla and thus may block sodium reabsorption. Taves et al (118) studied the inorganic fluoride and nonvolatile organic fluoride concentration in patients who developed methoxyflurane-associated kidney disease, and like Mazze, felt that inorganic fluoride ion is probably one of the more important responsible agents. In 1930, Goldemberg (119) reported a polyuric syndrome following the administration of sodium fluoride which was then used in the treatment of thyrotoxicosis. Roholm (120) suggested that polyuria is a prominent part of acute fluoride intoxication. Pindborg (121), Bond & Murray (122), as well as Taves et al (118) have described interstitial fibrosis in patients with history of long-term ingestion of fluoride.

Kosek et al (116) and Mazze et al (123) have conclusively demonstrated a physiological and anatomical renal tubular lesion in the rat identical to that seen in man. The lesion is proportional to methoxyflurane dosage and is not an idiosyncratic reaction. Inorganic fluoride ion formed by metabolism of the anesthetic agent appears to be responsible for the damage. From the same laboratory, Barr et al showed that methoxyflurane nephrotoxicity in the rat can be markedly aggravated by gentamicin, an event previously described by them in man (106). They have also shown that the metabolism of methoxyflurane and the susceptibility to the nephrotoxic effects varies in different strains of rats (115), but that there is a dose relationship to the nephrotoxicity (123).

Although oxalic acid has been implicated as a mechanism of nephrotoxicity and calcium oxalate crystals are found in the renal interstitia of patients with methoxyflurane renal failure, this may not explain the severe tubular dysfunction. In 1945, Jeghers & Murphy (124) reported that acute oxalate poisoning in man does not cause acute oliguric renal failure. Nor do we see a similar lesion acutely in patients with familiar hyperoxaluria (125). The patients with this familial disease show scarring, which occurs 40 to 50 years after the onset of deposition of calcium oxalate in the kidneys. Hollenberg et al (110) speculate that the critical factor lies in the metabolism of methoxyflurane into inorganic fluoride ion. They suggest that tubular obstruction with calcium oxalate may prevent recovery. The cortical flow is also reduced to levels that may be inadequate for the maintenance of glomerular filtration.

Aggravating factors in the methoxyflurane nephrotoxicity such as obesity were reported by Panner et al (108) in 2 fatal cases. Methoxyflurane is highly soluble in fat. Kuzucu (107) thought that tetracycline aggravated the onset of methoxyflurane renal disease. Mazze et al (105) reported gentamicin as aggravating the renal nephrotoxicity and demonstrated similar lesions in the rat. In the early cases of Paddock et al (104), surgical shock was implicated. However, in the case reports we have reviewed, this type of renal failure can be distinguished from the usual acute renal failure from surgery complicated by hypotension or mismatched blood transfusions. The onset of the renal failure occurred later than that expected after surgical shock—in most reported patients on the 10th or 12th postoperative day. Despite well-maintained urinary volumes, the characteristic and pathological lesion was the deposition of calcium oxalate crystals in the kidney. There also have been reports of inappropriate antidiuretic hormone syndrome following administration of methoxyflurane (100). This syndrome was also noted by Mazze et al (95) in their prospective study; they also reported this occurrence after halothane administration. However, there has been no report of halothane causing the syndrome of nephrogenic diabetes insipidus as described in methoxyflurane anesthesia. There have been no reports of cross-reactivity between these two agents producing renal failure. The explanation could be in the biotransformation of the two anesthetic agents in man. Methoxyflurane biotransformation has been shown (34) to result in the release of an inorganic fluoride ion, whereas this has not been shown to occur with halothane.

We conclude from these studies that there is a real risk to renal function in the administration of methoxyflurane, and that some fatal cases have been documented and well studied. This risk seems to be confined to methoxyflurane at the moment, although the newer fluorinated anesthetics have not yet been adequately studied. We also know that there was concomitant hepatocellular disease in some of the patients with renal disease (13, 80). In 1971, the Committee on Anesthesia of the National Academy of Sciences—National Research Council issued a special report on the evidence for the association of methoxyflurane anesthesia and renal dysfunction (126). They urged that the anesthetic continue to be available in clinical practice. Since that report, prospective studies have documented the nephrotoxic syndrome. The lesion has been reproduced in experimental animals and is dose-related. Fatalities have continued to be reported and their incidence is low perhaps because of the opportunity of recovery offered by renal dialysis and renal transplants. Although overall mortality from various anesthetic agents may be similar (3, 34, 99), we feel that the renal lesion should act as a deterrent to the use of methoxyflurane for prolonged surgery. Reasonable guidelines in relation to the prevention of severe renal effects have been suggested by Cousins & Mazze (97). Methoxyflurane should not be used in patients with pre-existing renal disease or given concurrently with other potential nephrotoxic drugs. In healthy individuals, the dose should not exceed 2–2.5 minimum alveolar concentration hours. Care in the use of accurate vaporizers and the measurements of renal function is necessary.

The known renal effects of methoxyflurane, as well as the reports of hepatotoxicity and massive hepatic necrosis and possible hepatic cross-sensitization with halothane, strongly suggest that the role of this anesthetic now needs careful reconsideration.

Literature Cited

1. Ngai, S. H. 1972. *Handb. Exp. Pharmacol. Sect. 3* 1:33–76
2. Van Dyke, R. A. 1972. *Handb. Exp. Pharmacol. Sect. 5* 2:347–55
3. Cascorbi, H. F. 1973. *Anesthesiology* 39:115–25
4. Cohen, E. N. 1971. *Anesthesiology* 35:193–202
5. Simpson, B. R., Strunin, L., Walton, B. 1971. *Brit. Med. J.* 2:96–100
6. Simpson, B. R., Strunin, L., Walton, B. 1973. *Proc. Roy. Soc. Med.* 66:56–63
7. Strunin, L., Simpson, B. R. 1972. *Brit. J. Anaesth.* 44:919–24
8. Dykes, M. H. M. 1973. *Can. Anaesth. Soc. J.* 20:34–47
9. Johnstone, M. 1956. *Brit. J. Anaesth.* 28:392–410
10. Burnap, T. K., Galla, S. J., Vandam, L. D. 1958. *Anesthesiology* 19:307–20
11. Virtue, R. W., Payne, K. W. 1958. *Anesthesiology* 19:562–63
12. Subcommittee on National Halothane Study of Committee of Anesthesia. National Academy of Sciences-National Research Council. 1966 *J. Am. Med. Assoc.* 197:775–88
13. Trey, C., Lipworth, L., Davidson, C. S. 1969. *Anesth. Analg.* 48:1033–42
14. Carney, F. M. T., Van Dyke, R. A. 1972. *Anesth. Analg.* 48:135–60
15. Dykes, M. H. M. 1970. *Int. Anesthesiol. Clin.* 8:241–313
16. Sherlock, S. 1971. *Gut* 12:324–29
17. Dundee, J. W., Miller, R. A. 1972. *Brit. J. Anaesth.* 44:909
18. McCaughey, W. 1972. *Brit. J. Anaesth.* 44:918
19. Dykes, M. H. M., Gilbert, J. P., McPeek, B. 1972. *Brit. J. Anaesth.* 44:925–34
20. Reed, W. D., Williams, R. 1972. *Brit. J. Anaesth.* 44:935–40
21. Davies, G. E., Holmes, J. E. 1972. *Brit. J. Anaesth.* 44:941–45
22. Farman, J. V. 1972. *Brit. J. Anaesth.* 44:946–52
23. Geddes, I. C. 1972. *Brit. J. Anaesth.* 44:953–60
24. Dundee, J. W., Miller, R. A. 1972. *Brit. J. Anaesth.* 44:909–70
25. Löfström, J. B. 1972. *Acta Anaesthesiol. Scand. Suppl.* 49. 43 pp.
26. *Proc. Roy. Soc. Med.* 1973. 66:55–63
27. *Can. Anaesth. Soc. J.* 1973. 20:1–120
28. Williams, R. 1973. *Proc. Roy. Soc. Med.* 66:63
29. Sharpstone, P., Medley, D. R. K., Williams, R. 1971. *Brit. Med. J.* 1:448–50
30. Combs, B. 1969. *N. Engl. J. Med.* 280:558–59
31. Stephen, C. R. 1969. *N. Engl. J. Med.* 280:561–62
32. Stier, A., Alter, H., Hessler, O., Rehder, K. 1964. *Anesth. Analg.* 43:723–28
33. Rehder, K., Forbes, J., Alter, H., Hessler, O., Stier, A. 1967. *Anesthesiology* 28:711–15
34. Van Dyke, R. A. 1973. *Can. Anaesth. Soc. J.* 20:21–33
35. Van Dyke, R. A., Chenoweth, M. B. 1965. *Biochem. Pharmacol.* 14:603–9
36. Brown, B. R. 1971. *Anesthesiology* 35:241–46
37. Biebuyck, J. F. 1973. *Anesthesiology* 39:188–98
38. Biebuyck, J. F., Lund, P., Krebs, H. A. 1972. *Biochem. J.* 128:721–23
39. Ibid 1972. 128:711–20
40. Smuckler, E. A., Bombeck, C. T., Nyhus, L. M. 1968. *Toxicity of Anesthetics,* ed. B. R. Fink, Chapt. 15, 176–85. Baltimore: Williams & Wilkins. 332 pp.
41. Van Dyke, R. A., Chenoweth, M. B., Van Poznak, A. 1964. *Biochem. Pharmacol.* 13:1239–47
42. Stenger, R. F., Johnson, E. A. 1972. *Proc. Soc. Exp. Biol. Med.* 140:1319–24
43. Paronetto, F., Schaffner, F., Popper, H. 1967. *J. Lab. Clin. Med.* 69:979–88
44. Paronetto, F., Popper, H. 1970. *N. Engl. J. Med.* 283:277–80
45. Rodriguez, M., Paronetto, F., Schaffner, F., Popper, H. 1969. *J. Am. Med. Assoc.* 208:148–50
46. Doniach, D., Roitt, I. M., Walker, T. G., Sherlock, S. 1966. *Clin. Exp. Immunol.* 1:237–62
47. Doniach, D. 1973. *Proc. Roy. Soc. Med.* 66:61–62
48. Cohen, A. B., Rosenthal, W. S., Stenger, R. J. 1973. *Proc. Soc. Exp. Biol. Med.* 142:817–19
49. Little, D. M. 1968. *Surv. Anesthesiol.* 12:406–8
50. Stenram, U. 1972. *Brit. Med. J.* 1:312
51. Dykes, M. H. M. 1971. *J. Am. Med. Assoc.* 216:641–44
52. Trey, C. et al 1968. *N. Engl. J. Med.* 279:798–801
53. Peters, R. L. et al 1969. *Am. J. Med.* 47:748–64
54. Uzunalimoglu, B., Yardley, J. H., Boitnott, J. K. 1970. *Am. J. Pathol.* 61:457–78

55. Dordal, E., Glagou, S., Orlando, R. A., Platz, C. 1970. *N. Engl. J. Med.* 283:357–59
56. Klion, F. M., Schaffner, F., Popper, H. 1969. *Ann. Intern. Med.* 71:467–77
57. Keeley, A. F. et al 1970. *Gastroenterology* 58:965
58. Mushin, W. W., Rosen, M., Bowen, D. J., Campbell, H. 1964. *Brit. Med. J.* 2:329–41
59. Slater, E. M., Gibson, J. W., Dykes, M. H. M., Walzer, S. G. 1964. *N. Engl. J. Med.* 270:983–87
60. Greene, N. M. 1973. *N. Engl. J. Med.* 289:304–7
61. Hollinger, F. B. et al 1973. *N. Engl. J. Med.* 289:385–91
62. Klatskin, G. 1968. *Toxicity of Anesthetics,* ed. B. R. Fink, Chapt. 14, 159–75. Baltimore: Williams & Wilkins. 332 pp.
63. Babior, B. M., Davidson, C. S. 1966. *Am. J. Med.* 41:491–96
64. Belfrage, S., Ahlgren, L., Axelson, S. 1966. *Lancet* 2:1466–67
65. Ahlgren, L. 1972. *Acta Anaesthesiol. Scand. Suppl.* 49:11–12
66. Klatskin, G., Kimberg, D. V. 1969. *N. Engl. J. Med.* 280:515–22
67. Mackay, P., Powell, L. W. 1968. *Med. J. Aust.* 1:68–71
68. Hughes, M., Powell, L. W. 1970. *Gastroenterology* 58:790–97
69. Davis, P., Holdsworth, C. D. 1973. *Gut* 14:566–68
70. Keeri-Szanto, M., Lafleur, F. 1963. *Can. Anaesth. Soc. J.* 10:531–38
71. Gall, E. A. 1968. *Anesthesiology* 29:233–48
72. Babior, B. M., Davidson, C. S. 1967. *N. Engl. J. Med.* 276:645–52
73. Brenner, A. I., Kaplan, M. M. 1971. *N. Engl. J. Med.* 284:961–62
74. Lindenbaum, J., Leifer, E. 1963. *N. Engl. J. Med.* 268:525–30
75. Katz, S. 1970. *Am. J. Dig. Dis.* 15: 733–39
76. Klein, N. C., Jeffries, G. H. 1966. *J. Am. Med. Assoc.* 197:1037–39
77. Lischner, M. W., McNabb, G. M., Galambos, J. T. 1967. *Arch. Intern. Med.* 120:725–28
78. Becker, F. P. 1970. *Lancet* 2:719–20
79. Judson, J. A., Dejongh, H. J., Walmsley, J. B. W. 1971. *Anesthesiology* 35:527–32
80. Elkington, S. G., Goffinet, J. A., Conn, H. O. 1968. *Ann. Intern. Med.* 69:1229–63
81. Joshi, P. J., Conn, H. O. 1973. *Gastroenterology* 65:A24–548
82. Reynolds, E. S., Brown, B. R., Vandam, L. D. 1972. *N. Engl. J. Med.* 286:530–31
83. Tucker, W. K. et al 1973. *Anesthesiology* 39:104–7
84. Harris, J. A., Cormwell, P. H. 1972. *Anesthesiology* 37:462–63
85. Johnston, R. R. et al 1973. *Anesthesiology* 38:313–19
86. Crandell, W. B., Pappas, S. G., MacDonald, A. 1966. *Anesthesiology* 27:591–609
87 Crandell, W. B., MacDonald, A. 1968. *J. Am. Med. Assoc.* 205:798–99
88 Gauert, W. B. et al 1969. *S. Med. J.* 62:1487–90
89 Lebowitz, M. H. 1969. *Anesth. Analg.* 48:233–36
90 North, W. C., Stephen, C. R. 1966. *Anesth. Analg.* 45:117–25
91 Austin, W. H., Villandry, P. J. 1967. *Anesthesiology* 28:637
92 Hook, J. B. 1971. *Anesthesiology* 35:238–40
93 Mazze, R. I., Trudell, J. R., Cousins, M. F. 1971. *Anesthesiology* 35:247–52
94 Mazze, R. I., Cousins, M. J. 1973. *Can. Anaesth. Soc. J.* 20:64–80
95 Mazze, R. I., Shue, L., Jackson, S. H. 1971. *J. Am. Med. Assoc.* 216:278–88
96 Tobey, R. E., Clubb, R. T. 1973. *J. Am. Med. Assoc.* 223:649–52
97 Cousins, M. J., Mazze, R. I. 1973. *J. Am. Med. Assoc.* 225:1611–16
98 Pezzi, P. J., Frobese, A. S., Greenberg, S. R. 1966. *Lancet* 1:823
99 Urgena, R. B., Gergis, S. D. 1973. *Brit. J. Anaesth.* 45:358–61
100 Black, G. W., Keilty, S. R. 1973. *Brit. J. Anaesth.* 45:353–57
101 Wilson, T. 1973. *Brit. J. Anaesth.* 45:232
102 Latto, I. P., Wainwright, A. C. 1973. *Brit. J. Anaesth.* 45:232–34
103 Artusio, J. F. et al 1960. *Anesthesiology* 21:512–17
104 Paddock, R. B., Parker, J. W., Gaudagni, N. P. 1964. *Anesthesiology* 25:707–8
105 Mazze, R. I., Cousins, M. J. 1973. *Brit. J. Anaesth.* 45:394–98
106 Barr, G. A., Mazze, R. I., Cousins, M. J., Kosek, J. C. 1973. *Brit. J. Anaesth.* 45:306–11
107 Kuzucu, E. Y. 1970. *J. Am. Med. Assoc.* 211:1162–64
108 Panner, B. J. et al 1970. *J. Am. Med. Assoc.* 214:86–89

109. Frascino, J. A., Vanemee, P., Rosen, P. P. 1970. *N. Engl. J. Med.* 283: 673–79
110. Hollenberg, N. K. et al 1972. *N. Engl. J. Med.* 286:877–79
111. Halpren, B. A., Kempson, R. L., Coplon, N. S. 1973. *J. Am. Med. Assoc.* 223:1239–42
112. Frascino, J. A. 1972. *J. Lab. Clin. Med.* 79:192–203
113. Aufderheide, A. C. 1971. *Arch. Pathol.* 92:162–66
114. Holaday, D. A., Rudofsky, S., Treuhaft, P. S. 1971. *J. Am. Med. Assoc.* 214:91–95
115. Mazze, R. I., Cousins, M. J., Kosek, J. C. 1973. *J. Pharmacol. Exp. Ther.* 184:481–88
116. Kosek, J. C., Mazze, R. I., Cousins, M. J. 1972. *Lab. Inv.* 27:575–80
117. Mazze, R. I., Cousins, M. J. 1973. *Can. Anaesth. Soc. J.* 20:64–80
118. Taves, D. R. et al 1970. *J. Am. Med. Assoc.* 214:91–95
119. Goldemberg, L. 1930. *Rev. Soc. Med. Int. Soc. Tisiolog* 6:217–42
120. Roholm, K. 1937. *Fluorine Intoxication,* 262–68. London: Lewis
121. Pindborg, J. J. 1957. *Acta Pharmacol. Toxicol.* 13:36–45
122. Bond, A. M., Murray, M. M. 1952. *Brit. J. Exp. Pathol.* 33:168–76
123. Mazze, R. I., Cousins, M. J., Kosek, J. C. 1972. *Anesthesiology* 36:286–92
124. Jeghers, H., Murphy, R. 1945. *N. Engl. J. Med.* 233:208–15
125. Wyngaarden, J. B., Elder, T. D. 1966. *The Metabolic Basis of Inherited Disease,* ed. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, 189–212. New York: McGraw
126. Committee on Anesthesia. National Academy of Sciences—National Research Council. 1971. *Anesthesiology* 34:505–9

Copyright 1974. All rights reserved

THE CELL SURFACE AND MALIGNANT TRANSFORMATION

◆7123

R. E. Pollack, Ph.D. and P. V. C. Hough, Ph.D.
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, and Biology Department, Brookhaven National Laboratory, Upton, New York

A malignant cell has lost many restrictions to which normal cells are subjected. For example, cell division is no longer tightly regulated by cell-cell contact or specific serum factors, serum-mediated hormonal feedback loops are broken, and cell recognition of neighboring cells no longer leads to maintenance of cell position.

As such differences (Table 1) between normal and malignant cells are described in ever greater detail, mechanisms to explain how the cell maintains any or all of them have remained sorely lacking.

Very recently, different laboratories working on widely different biological systems have reported a series of experiments (1–7) which, taken together, begin to suggest possible mechanisms for the maintenance of the transformed state. Most of these new lines of research have directly involved experiments on the surfaces of normal and malignant cells.

In this review we have attempted to make explicit some implied connections among the results of these laboratories. Our hope is that our admittedly premature synthesis might be predictive, and so stimulate some new critical experiments.

STRUCTURE OF THE CELL SURFACE

In normal and transformed cells alike the cell surface acts both as a transmitter and as a transducer of signals from other cells and from serum or culture medium. The signals received by the surface may be virus-induced gene products [e.g. the proteolytic activity of Reich and co-workers (6)] or hormones [e.g. insulin (8)]; and output signals may be issued to the interior of the cell via membrane-bound enzyme systems [e.g. adenylate cyclase (9)], or the active transport systems (10), or possibly by changing the attachment of membrane proteins and glycoproteins to a postulated internal structure involving microtubules (11).

A widely accepted current view of the molecular structure of the surface consistent with those functions is illustrated in Figure 1 (12, 13). The membrane is a lipid bilayer with hydrophobic hydrocarbon tails 14–24 carbons long in the interior, and either (*a*) polar head groups linked by phosphate or (*b*) polysaccharide structures of variable complexity at the outer surface. The membrane thickness has been determined by small angle X-ray diffraction to be 46 Å (14).

Membranes from different tissues contain varying amounts of the rigid molecule, cholesterol, (15) and it has been argued that parts of the membrane may be in a gel or crystalline state (16). Recent studies of the membrane-bound enzyme adenylate cyclase (17) show a discontinuity in activation energy for the enzyme at 32° C, and while this temperature is nonphysiological, the unexplained effect suggests that we should expect complex interactions between the enzymatically active proteins of the membrane and the lipid matrix.

It is most promising to look to the protein and glycoprotein component, the embedded globular particles of Figure 1, for the control and signal properties of the membrane. Experiments with intact erythrocytes and erythrocyte ghosts (18–21) establish, for the red blood cells at least, that while most membrane proteins and glycoproteins are exposed only at the inner surface, a major protein and a major

Table 1 Properties of cells transformed by DNA or RNA tumor viruses

Growth	
	High or indefinite saturation density
	Reduced serum requirement
	Growth in agar or methocel suspension
	Tumor formation on injection into susceptible animals
	Reduced contact inhibition of movement
	Disoriented growth pattern
	Growth on monolayers of normal cells
Surface	
	Increased agglutinability by plant lectins
	Changes in composition of glycoproteins and glycolipids
	Virus-induced transplantation antigen
	Increased rate of transport of nutrients
Enzymatic	
	Production of cell factor component of fibrinolysin T
	Increased anaerobic glycolysis
Evidence of Virus	
	Virus-specific antigens present
	Virus DNA or RNA sequences detected
	Virus rescued by heterokaryon formation

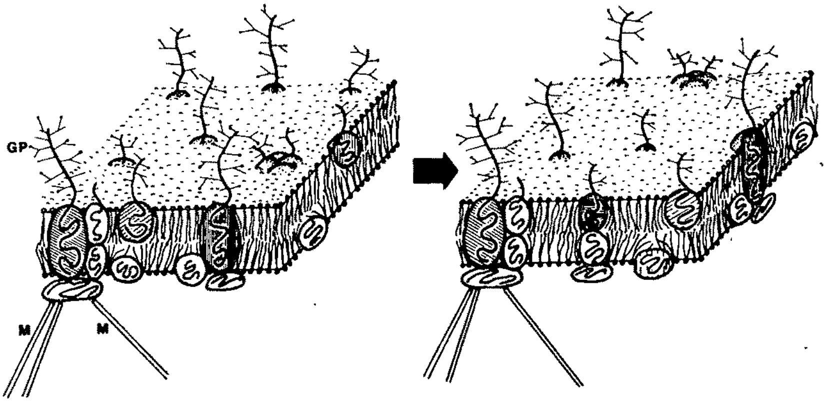


Figure 1 The fluid mosaic model of the cell membrane (12) adapted from Singer & Nicolson (13) with hypothetical cytoplasmic restraints. Some membrane components (glycoproteins, glycolipids, and lipids) are rapidly diffusing in the membrane plane under physiological conditions (left sketch → right sketch), while others such as the glycoprotein complex (*GP*), illustrated at the left, have their mobility impeded by membrane-associated interior cellular structures (*M*). Used with permission from *Control of Proliferation in Animal Cells,* Cold Spring Harbor Laboratory, 1973.

glycoprotein penetrate the lipid bilayer and are exposed at both the inner and outer surfaces of the cell. In Bretscher's experiments (19, 20), for example, the labeling reagent [^{35}S] formyl-methionyl (sulfone) methyl-phosphate was used to add formyl-methionyl sulfone groups to any exposed amino (and possibly hydroxyl) groups on the membrane particles. Exposing intact erythrocytes only labels groups on the outside surface of the membrane, while exposing lysed ghost cell membranes permits labeling of both outside and inside groups. By this technique, Bretscher showed that a single major 100,000-dalton protein was exposed on both surfaces of the red cell membrane. To prove that some parts of the protein are outside and others inside, Bretscher (20) isolated the protein and prepared a "fingerprint" of its tryptic digest. Only a few spots of the fingerprint appeared when the protein was labeled in intact cells but a more complete fingerprint, which included the earlier spots, appeared when ghosts were labeled.

The freeze fracture technique (22) cleaves the membrane at a midplane between the lipid layers. Electron microscopy of the newly exposed previously interior surfaces of the membrane reveals particles (23, 24); since the sizes of the particles are as predicted from membrane protein molecular weights (24), the number of particles (24) agrees with the number predicted from chemical analysis (19), and the particles observed by electron microscopy are digested by proteases (25, 26), we can conclude that the observed particles are the membrane proteins and glycoproteins. Usually the particles are found randomly distributed laterally in the membrane, but e.g. in *Tetrahymena,* Satir et al (27) find that a rosette of particles appears at the point where a mucocyst begins to merge with the cell membrane. It will perhaps not be surprising to find that over the next few years such phenomena as nutrient and ion transport, agglutination, and cell division are successfully accounted for in terms of the composition and distribution of membrane particles and their connections to the interior of the cell.

Whatever their function, membrane glycoproteins appear to be free to move about rapidly in the lipid bilayer, giving the membrane as a whole the somewhat paradoxical properties of transmembrane impermeability and lateral fluidity. Frye & Edidin (28) and Edidin & Weiss (29) have obtained direct evidence for lateral mobility of membrane glycoproteins and proteins in mouse and human fibroblasts. Cells of a mouse cell line and a human strain were fused with the aid of inactivated sendai virus. Mouse H2 antigens (glycoproteins) and human species antigens (believed to be proteins) were localized at light microscopic resolution on the surface of the fused cell with fluorescent antibodies. Immediately after fusion the two groups of antigens were segregated. The number of cells with segregated antigens then decreased exponentially with a half-life dependent on temperature, but not on generation of ATP or on protein or amino sugar synthesis in the cell. These data suggest that the mixing of antigens results from simple diffusion. In view of the drastic effect of sendai virus on the membrane in promoting fusion one may worry about applying this result to normal cells, but under the conditions of the experiment it is clear that membrane proteins and glycoproteins are free to move laterally.

Lateral fluidity was related to the general problem of transformation when Edidin and co-workers showed that the mobilities of the particular surface antigens they detect were far higher in transformed than normal cells. They measured a half-life of only 20 min for intermixing in the case of fusion of a mouse L cell with an SV40-transformed human fibroblast, but about a 2-hr half-life for fusions of 3T3 with secondary human fibroblasts, and of a revertant line (30) with the untransformed diploid strain of human fibroblasts WI38. The question of mobility of membrane particles will come up again below when we discuss agglutinability. This property shows every sign of developing into a major clue to the nature of transformation.

As yet, no compelling functional role in transformation has been devised for the glycolipids and we will somewhat reluctantly pass them by, making reference to chapter 3 of Tooze (15).

TWO MISSING LINKS

Normal cells in vivo or in culture may, during the G_1 phase of the cell cycle, elect or be induced to enter a differentiated resting state usually called G_0. Cells in G_0 can be induced to reenter the cell cycle by a variety of agents, many acting at the cell surface.

Therefore, we consider missing link I in our understanding of control of proliferation to be the sequence of biochemical events occurring between stimulation of the surface and initiation of DNA replication. We devote the following two sections to presumed components of this signal system. Of course, some inducers of departure from G_0 such as replacement of a missing amino acid for a starved culture or supply of Ca^{2+} to the G_0 chick fibroblast system of Balk et al (31) are not likely to act directly on the membrane. Accordingly, our first missing link is really a missing network of which one link includes the surface and a culminating link initiates synthesis of a critical precursor to DNA replication. In view of the variety of input effectors, the link at which the network yields to analysis may be the culminating and not the surface link.

Missing link II is the sequence of biochemical events leading from the integration of the genome of the oncogenic virus into the host chromosome to modification of "the surface," or (as a realistic generalization) to modification of the proliferation control network which we have just named missing link I. We devote the last three sections of this review to missing link II. In the section on viral transformation we discuss the changes in growth, surface, and other cell parameters associated with transformation and consider briefly two useful virus-cell systems. In the last sections we discuss two cellular effects that are perhaps as close to the transforming virus as we have yet come. One effect is the freeing of some surface receptors that are immobilized in untransformed cells; the second is the induction of a proteolytic activity which probably acts at the cell surface and may have the effect of freeing the receptors.

CYCLIC NUCLEOTIDES

Cyclic AMP (3',5'-adenosine monophosphate) is manufactured from ATP by an enzyme of the plasma membrane (32), adenylate cyclase, and reclaimed to 5'-AMP by phosphodiesterase. A decrease in diesterase activity or an increase in cyclase activity or both will lead to an increase in cyclic AMP. Epinephrine and glucagon in the liver and muscles act by stimulating adenylate cyclase, but in the wide assortment of physiological processes mediated by cAMP (32), both enzymes are important.

Cyclic GMP (3',5'-guanosine monophosphate) was discovered by Goldberg and co-workers (1) in the late 60s in many animal tissues at a concentration one to two orders of magnitude less than that of cAMP. The first correlation of cGMP with physiological function was found (33) in cardiac contractility in the rat, stimulated by isoproterenol and depressed by acetylcholine, and accompanied by a decrease and increase, respectively, in the level of cGMP. In many cases cGMP concentration is altered in a sense opposite to that of cAMP. Many other hormone-induced changes in cell function have since been found to be correlated with cGMP level (34).

The correlation of cAMP level with cell proliferation has been extensively investigated by Pastan (35), Sheppard (36), and many others. There is general agreement that the concentration of cAMP in cells showing reduced density-dependent inhibition of growth is about one half the concentration in related normal cells. Also, it is generally agreed that agents that induce proliferation in resting cultures, e.g. fresh serum, insulin, and trypsin, all reduce cAMP concentration, while serum depletion raises it. Concerning the important question of change in cAMP concentration as a cell population passes from the log phase to the stationary phase of growth, Sheppard (37) found no change for 3T3 mouse fibroblasts, while Pastan and co-workers (35, 38–40) found a marked increase not only for 3T3 cells but also for normal rat kidney (NRK) cells and human fibroblasts. In NRK cells they found a more than 7-fold increase in cAMP concentration due to a large increase in adenylate cyclase activity overcompensating a minor increase in phosphodiesterase activity. In interpreting these experiments (35) it is important to be sure that serum deprivation did not inadvertently occur as cultures reached confluence (37).

In any event all such experiments are consistent with the hypothesis that a modulation of the activity of membrane-bound adenylate cyclase may control proliferation by causing a net elevation in the level of cAMP.

Temin (8) has shown that insulin stimulates cell division. An accompanying decrease in cAMP concentration was found by Sheppard (37), and very recently, Goldberg and co-workers (34) found in 3T3 cultures an accompanying increase in cGMP concentration—about 10-fold within 3 min at an insulin concentration of 10 mU/ml. Additional provocative but as yet isolated observations include Estensen et al's (41) finding that within 60 sec the tumor promoter phorbol myristate acetate, which induces cell division in 3T3 fibroblasts, produces a 10-fold increase in cellular cGMP concentration; also cGMP concentrations between about 10^{-8} and 10^{-11} M stimulate [^{3}H] uridine incorporation into RNA by isolated lymphocyte nuclei (36).

It is clear that the molecular biology of the control of adenylate and guanylate cyclase must be pursued intensively, and a search made for enzymes influenced by the cyclic nucleotides and connected with the cell's commitment to DNA synthesis. Whether one of the two purine cyclic nucleotides is master and the other slave, or whether their two cyclases have a common modulator, or whether the interaction yielding a frequently contrary change in cGMP and cAMP levels is more complex is so far unknown. Since the distinctive association of both cyclic nucleotide concentrations with the state of cell proliferation persists for the case of viral transformation, it seems certain that much more of this story will be written over the next few years.

GROWTH STIMULATING FACTORS

If the saturation density of 3T3 cells in culture is plotted against the serum concentration in the nutrient medium, the resulting smooth curve continues without break in slope through and well beyond the point of confluence (42). Holley and Kiernan (42, 43), Temin and co-workers (44–46), and others have concluded from such data that nutrient or control factors in serum or medium are the determining elements for proliferation and that contact-dependent inhibition of division is not per se a separate phenomenon. These workers are looking in depth for growth stimulating factors and the functional role of such factors. One recent product of the search is Multiplication Stimulating Activity (MSA) purified originally from calf serum by Pierson & Temin (44).

The relation of MSA to other such factors is only now becoming clear. Over the past 15 years, studies of growth stimulating factors in human serum have led to the partial purification of several activities originally named simply for their function. One is "sulfation factor" (47), which stimulates the uptake of [^{35}S]O$_4$ by cartilage;

another is "nonsuppressible insulin-like activity" [see (48) and references therein], which stimulates incorporation of thymidine in DNA, incorporation of amino acids into rat diaphragm, and a number of other insulin-like activities. This activity is different from that of insulin because it is not suppressed by antiserum to insulin. Apparently, all these activities in human serum may be ascribed to a single small protein (mol wt $\sim$ 7000) named somatomedin (48). Hintz et al (48) have shown that somatomedin competes with insulin for binding to receptors which in turn have been shown by Cuatrecasas (49) to reside on the cell surface.

MSA, purified recently some 35,000-fold relative to MSA in calf serum by Dulak & Temin (45, 46), is identified as a small protein (mol wt $\sim$ 10,000), which is found to have the sulfation factor and nonsuppressible insulin-like activities of somatomedin. MSA, somatomedin, and insulin all compete for the same receptors [Van Wyk, quoted by Temin (2)]. It is therefore entirely possible that MSA and somatomedin are the same molecule.

The source of the recently purified MSA is itself of interest. A line of untransformed buffalo rat liver cells isolated by Coon (50) has the rare ability to grow in serum-free medium. Medium conditioned by the rat liver cells is the starting point of the purification. Thus, at least some cells can produce their own MSA.

MSA substitutes for serum, at a protein concentration 10^{-5} that of the serum, in inducing the incorporation of thymidine into chick embryo cell DNA as measured in a 1-hr pulse 12 hr after stimulation. It also substitutes for serum in supporting proliferation of untransformed chicken and duck embryo fibroblasts, but must be supplemented with 0.1% serum to support chick cells infected by Rous sarcoma virus (RSV).

VIRAL TRANSFORMATION

The transformation of animal cells in culture by oncogenic viruses initiates a broad spectrum of changes in cell behavior. For a review of transformation, see chapters 6 and 11 of reference 15. A comprehensive though not complete list of the properties distinguishing transformed cells from their untransformed parent cells is given in Table 1. The phenomenology of transformation is based largely on the study of infection of mouse, hamster, and monkey cells by the DNA-containing papova viruses SV40 and polyoma, and infection of chicken, mouse, and rat cells by the RNA tumor viruses, Rous (or avian) sarcoma virus (RSV), murine sarcoma virus (MSV), and associated RNA helper viruses, avian and murine leukosis virus (ALV and MLV). It is interesting that Table 1 applies to transformation by both DNA and RNA tumor viruses, and that more than half of the list is associated either directly or by implication with changes in the cell surface.

While it is known that in transformation the viral genome is retained and replicates in the cell nucleus (51–53), and is most probably integrated in the cell genome (54), no biochemical mechanism by which the transforming virus exerts its effect on the cell has yet been identified. For our present purpose of elucidating the effect of viral transformation on the cell surface and on proliferation, we will first describe an experiment with SV40 that demonstrates the range and variability of viral effects, and then focus on the new experimental system of temperature-sensitive (*ts*) avian sarcoma viruses in interaction with chick fibroblasts, which has provided clear answers to a few basic questions on the nature of transformation.

The properties of transformed cells listed in Table 1 can in many cases be used for selection of transformants following viral infection. Thus, mouse 3T3 cells transformed by SV40 can be isolated by recloning from colonies observed to grow to higher than normal density after infection, in nutrient medium containing a standard 10% concentration of calf serum. For study of the frequency and range of expression of the various characters of the transformed phenotype, however, a nonselective assay is required; such a study has recently been carried out by Risser & Pollack (4). Following infection of 3T3 cells by SV40 virus at a concentration of 2 X 10^3 infectious units per cell, some 40 clones were picked at random, i.e. without regard to growth properties or morphology. If the random clones are assayed for frequency of transformation as defined by abnormally high saturation density in 10% serum, only 25% of the clones are transformed. Such a result has typically led in the past to the statement that SV40 transformation of 3T3 is a low frequency event. However, if transformation is defined as the expression of any of the ensemble of properties in Table 1, fully 90% of the clones are found to be transformed. The property of serum transformation, i.e. the ability of a clone to proliferate in 1% serum with the 30-hr doubling time observed for standard high density transformants, is especially interesting. In the present experiment, serum transformation is exhibited by all clones that are transformed in any other assay, and also by some clones that are normal by all other assays, and therefore may be considered to be the fundamental property of transformation. Other parameters of transformation in Table 1 show a broad range in degree of expression. Thus SV40 T-antigen is not detected by fluorescein-coupled antibodies or complement fixation in some 40% of the clones, and an additional 25% show mixed positive and negative fluorescent labeling from cell to cell of a clone.

We conclude from this experiment that the interaction of virus and cell in transformation is far from a simple all or none switch. However, the ability of transformed cells to grow in low serum may be primary, and it may be possible in the future to relate such a primary parameter of transformation to the structural and enzymatic effects discussed in the following sections.

The infection of chick fibroblasts by members of the rapidly growing family of *ts* mutants of avian sarcoma virus is a promising current system for discovering mechanisms of viral transformation [for review see Vogt et al (55)].

To recover *ts* mutants, virus stocks are mutagenized by treatment with nitrosoguanidine or by permitting replication in the presence of the RNA mutagens 5-azacytidine or 5-fluorouracil or the DNA mutagen 5-bromodeoxyuridine. Most mutant viruses have been isolated by random cloning and testing of infected cells. Since the first isolations by Martin (5) and Biquard & Vigier (56) in 1970, several dozen temperature-sensitive mutants have been prepared by ten or more groups (see reference 15). All of these mutants are permissive, i.e. cause transformation, at 35–37° C and are nonpermissive at 40–41° C. In general, temperature sensitivity is believed to result from reduced conformational stability of an aberrant protein coded by a mutant gene. Thus we can already conclude that chick fibroblasts are transformed by avian sarcoma virus as a result of the positive action of a virally coded protein on the cell system.

For a class of *ts* viral mutants called T1 (57), maintenance of the transformed cell phenotype requires maintenance of the permissive temperature. Thus some viral gene products are required continuously for cell transformation. The following membrane-specific properties of transformed cells (Table 1) are among those made temperature sensitive by T1 mutants: increased rate of sugar uptake, increased agglutinability by plant lectins, changes in the amount of particular glycolipids and glycoproteins in the cell membrane, reduced concentration of cAMP, and production of fibrinolysin T (6).

The time course and requirements for RNA and protein synthesis in establishing the transformed phenotype on temperature shift down or the normal phenotype on shift up show interesting variations for the different T1 mutants. For some, 50% transformation occurs in 5–6 hr and requires protein synthesis but not RNA synthesis (58, 59). The return to normal phenotype occurs in the same time and requires no macromolecular synthesis at all (59). For the mutant of Bader (60) morphological transformation appears within 1 hr, even in the presence of inhibitors of protein synthesis. For the T5 mutant of Martin (61) the appearance of fibrinolysin T (6) seems to require both RNA and protein synthesis.

The genetics of the temperature-sensitive viruses has barely begun, but already Wyke (62) has divided a group of 11 T1 viruses into four complementation groups on the basis of their ability to complement one another in producing transformation at the nonpermissive temperature. This result, if taken at face value, implies at least four distinct viral genes for RSV transformation.

The *ts* avian sarcoma viruses so far discussed are temperature-sensitive for transformation but not for virus production. Some mutants are coordinately *ts*, however, and one such, *ts*334, has unusual transformation properties. It is temperature-sensitive for morphological transformation, as shown by the scanning electron micrographs of Figure 2, but not for growth in agar suspension culture (63). Thus the opportunity may exist for examining separately the genetic and biochemical basis for these two phenotypic characters.

AGGLUTINATION, SURFACE RECEPTORS, AND INTERNAL CONNECTIONS

Since the discovery by Ambrose et al (64) and Aub et al (65) of the preferential agglutinability of cells from spontaneous tumors by some plant lectins, Burger and co-workers (66, 67), Inbar & Sachs (68), and others have established a strong correlation between loss of density-dependent inhibition of growth for cells in culture and an increase in agglutinability. However, using the lectin concanavalin A (conA), labeled with ^{125}I or ^{3}H, Arndt-Jovin & Berg (69), Cline & Livingston (70), and Ozanne & Sambrook (71) found that increased agglutinability was not associated with a significant increase in the amount of lectin bound per cell. Nicolson

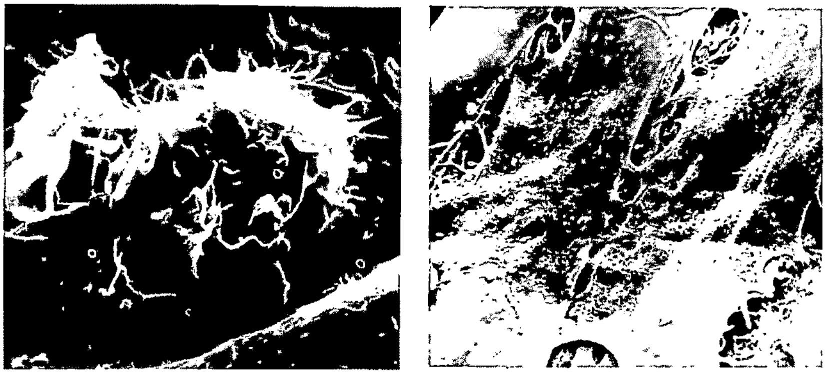


Figure 2 Chick cells infected with *ts*334, a temperature-sensitive mutant of avian sarcoma virus B77. (*left*) At permissive temperature (35° C), cell transformed; (*right*) at nonpermissive temperature (41° C), cells not transformed. Magnification X 5000. Scanning electron micrographs by A. Boyde and R. Weiss. Used with permission from J. Tooze, Ed., *The Molecular Biology of Tumor Viruses* (15).

(72) pointed the way to resolution of this puzzle by electron microscopy. The distribution of conA bound to 3T3 cells and SV40-transformed SV3T3 cells was observed using ferritin-conjugated conA. On agglutinable cells conA was found in patches, and on nonagglutinable cells it was found randomly dispersed.

The question then arises whether conA receptors on agglutinable cells preexist in patches or whether the patches are produced by the addition of the conA. The work of Rosenblith et al (73) suggests the latter. These workers used electron microscopy of shadow-cast replicas of 3T3 and SV3T3 surfaces; the positions of conA receptor complexes were marked by hemocyanin, a molecule 350 Å in diameter which binds specifically to conA. They observed that aggregates are not formed on either 3T3 or SV3T3 surfaces at 4° C, and also not at 37° C when the cells are prefixed by 1% paraformaldehyde before the addition of conA. This result demonstrates that conA receptors are dispersed on both agglutinable and nonagglutinable cells before addition of conA, and that reduction of the temperature to 4° C or prefixation immobilizes the receptors.

At 37° C and without prefixation, agglutinable SV3T3 cells form patches while nonagglutinable 3T3 cells do not, as had been shown by Nicolson (72). These results, taken together, demonstrate that conA receptors of transformed cells have lateral mobility but that receptors of untransformed cells are not free to move, even in the absence of any experimental conditions designed to fix them.

The conclusions so far are independent of any model, but it is natural to make the further interpretation that patches are formed when laterally diffusing receptors approach each other closely enough to be cross linked by conA. Native conA has four binding sites for α-methylglucose-like receptors (74) and therefore can in principle form networks of receptors just as antibodies form networks with antigen.

The original interpretation of Nicolson (72), that agglutination of cells results from the concentrated cell-cell binding force which patches can provide, still holds. However, since the main interest in agglutination is in the insight it can provide about surface receptors, we do not need to insist on a connection between patches and agglutination and can instead work directly with the results concerning receptors of a single cell.

It is well known that 3T3 cells are made as agglutinable as SV3T3 cells by treatment with trypsin (75). This result is both explained and made more significant by further experimental results of Rosenblith et al (73). They observe that trypsinized 3T3s form patches of conA at 37° C, but not at 4° C or when prefixed. Thus trypsin appears to act by freeing 3T3 conA receptors of their normal constraints, as does transformation by SV40. The reciprocal question whether viral transformation frees conA receptors by proteolytic activity is not yet answered, but in view of the proteases found to be associated with transformed cells (see the next section), experiments to answer the question will undoubtedly be performed soon.

Note that the earlier view that trypsin produces agglutinability by exposing lectin receptors (which however disagreed with experiments showing no increase in lectin binding) may now be replaced by the idea that trypsin removes normal cellular restraints on receptor mobility. Since we have given evidence above that some membrane particles are free to move, the source of this restraint becomes of great interest. We will present a hypothesis below but first consider some parallel evidence from lymphocytes.

Taylor et al (76) discovered that when the immunoglobulin-bearing surface of the lymphocyte is exposed to fluorescein conjugated antiimmunoglobulin antibodies, the endogenous immunoglobulin, which we will call "receptor Ig," first aggregates into patches that are subsequently transported to one pole of the cell (to form a "cap") and then engulfed. Patch and cap formation has been confirmed and characterized further by de Petris & Raff (77), Edelman and co-workers (78–80, 82), and Unanue, Perkins & Karnovsky (81).

The plant agglutinin conA is also bound to lymphocyte surfaces. This fact has permitted a determination whether conA receptor sites on lymphocytes also cluster. In fact, if conA is added to lymphocytes at 4° C, excess conA washed away, and the cells brought to 37° C, conA forms patches and caps with its own receptors (78, 80). However, if conA is added at 21 or 37° C, then both its own patch and cap formation and that of receptor Ig are inhibited. Both inhibitions are surprising. The conA self-inhibition is surprising because the previous 4–37°C incubation experiment shows that conA receptors on lymphocytes can be mobile under some conditions. The cross inhibition between conA and receptor Ig is surprising because the two sets of receptors are certainly different and neither one occupies a large enough fraction of the membrane surface to make it likely that there can be any simple steric interference of one group with the other.

An additional observation by Edelman, Yahara & Wang (79) may point to an explanation: both the self and cross inhibitions are reversed by colchicine, colcemid, vinblastine, and vincristine—four reagents which promote the disassembly of microtubules (83).

Therefore, Edelman and co-workers (11) hypothesize that there is an interior cellular structure of microtubules bound to various groups of membrane receptor proteins and glycoproteins and that the binding can be modulated, i.e. promoted by some reagents or physical conditions and diminished by others. They suggest that conA may stimulate the internal structure to bind to both conA receptors and receptor Ig and that such binding prevents lateral mobility of both sets of receptors. How would the evidence for free conA receptors after 4–37° C incubation be explained? Microtubules can dissociate at low temperature (84) and may reassemble at 37° C only on a time scale of hours (85); therefore patch and cap formation of conA receptors may occur before the internal structure is restored.

The interesting possibility that the microtubular structure is involved in the first steps of mitogenesis arises as follows: some lectins such as conA are mitogenic and others such as wheat germ agglutinin are not. Preliminary experiments reported by Edelman (11) show that a number of mitogenic lectins inhibit receptor Ig cap formation and a number of nonmitogenic lectins do not. Thus the mitogenic lectins may be precisely those having receptors that can be attached to the internal immobilizing structure. In support of this view, mitogenesis by conA is inhibited by colchicine, vinblastine, and vincristine at low concentration (11).

A PROTEOLYTIC ACTIVITY QUITE GENERALLY ASSOCIATED WITH TRANSFORMED CELLS IN VITRO AND MALIGNANT TUMORS IN VIVO

Reich and co-workers (6, 7) have recently discovered a proteolytic activity which they have shown to be broadly associated with transformed cells in vitro and in vivo. An early and previously neglected clue leading to the discovery was found in the cell culture experiments of Fischer (87) where explanted tumors but not bits of normal tissue were observed to lyse a blood clot used as substrate. Repeating Fischer's experiments under conditions of modern cell culture, Unkeless et al (6) and Ossowski et al (7) found that virus-transformed fibroblasts but not normal fibroblasts hydrolyze a substrate of fibrin. Fibrinolysis is induced by both DNA- and RNA-transforming viruses but not by a wide range of cytolytic viruses or the nontransforming avian leukosis viruses or the temperate paramyxovirus SV5 (6, 86). As noted earlier, the production of fibrinolytic activity is temperature sensitive (6) in chick cells infected with Martin's *ts* mutant of avian sarcoma virus, T5 (61). The activity can be detected within 1 hr of shift to permissive temperature (86) and, therefore, is an early event in transformation. Fibrinolysis is observed to be associated with primary cultures of chemically induced mammary carcinomas, hepatomas, and skin cancers and with a variety of human and animal tumor cell lines (86). It is not associated with the single class of benign tumor so far tested, the rat mammary fibroadenoma (86).

Reich and co-workers determined (6, 7) that the fibrinolytic activity, which they name fibrinolysin T (for tumor), arises from the coordinate activity of two proteases. One, T_S, is present in all vertebrate sera (6, 7, 86) and has been identified by Quigley et al (88) as the known zymogen, plasminogen (mol wt 90,000). The second protease,

T_C, was shown to be present in trace amounts in transformed cells only. T_C has been partially purified and characterized by Unkeless et al (89) as an arginine-specific protease (mol wt 38,000); it hydrolyzes a single peptide bond in plasminogen to generate the active fibrinolytic protease, plasmin (EC 3.4.4.14).

T_C from a given transformed cell type will not produce fibrinolysis with all sera. Thus, T_C (6) from RSV-infected chick embryo fibroblasts produces fibrinolysis with chicken and monkey sera but not with dog or hamster sera, while T_C (7) from SV40-transformed hamster cultures produces fibrinolysis with dog, hamster, and monkey sera but not with chicken or calf sera. The serum specificity is in part due to inhibitors of plasmin in some sera (86), but is also in part due to a specificity of interaction of the particular T_C with the plasminogen of the particular serum. Thus the failure of T_C from transformed hamster cells to produce fibrinolysis with chicken serum is explained by the observation (88) that plasminogen from chicken serum is not activated by hamster T_C.

While the interactions of T_C, T_S, and serum inhibitors are not yet fully understood, the existence of serum specificity can nevertheless be used as an experimental tool. For example, the spectrum of active sera (7) for mouse cells is the same whether the mouse cells are transformed by SV40 or MSV, but different from the spectrum for hamster cells transformed by SV40. T_C is therefore characteristic of the cell type and not of the transforming virus. Whether T_C is newly synthesized following transformation or merely activated is not currently known, but T_C is found in homogenates of transformed cells, tightly bound to one of the sedimentable membranous cellular particulates in the postnuclear fraction (86).

Initial studies by Ossowski et al (7, 90, 91) indicate that some of the parameters of transformation listed in Table 1 are determined, at least in part, by the activation of the fibrinolytic proteases. Reich and co-workers suggest that characteristic changes of cell morphology (7, 90), colony formation in soft agar (91), and enhanced cell mobility (91) all require the presence of an intact fibrinolytic system. They show also (91) that transformed cells produce receptors for plasmin which are not detectable in normal cultures. The relationship (if any) between fibrinolysis and saturation density of cultures is under study (86).

Considering a possible role for the fibrinolytic system in the formation and growth of tumors in vivo, Reich (86) reasons that the initiation of growth by either normal or transformed cells may require an increased level of plasma protein over that normally found in the extravascular, extracellular fluid compartment. He cites as an example an increase by nearly two orders of magnitude in the concentration of plasma protein in the cerebrospinal fluid of patients with brain tumors, and speculates that fibrinolysin T may produce effects of this type by increasing the permeability of the vascular system.

We may speculate further that fibrinolysin T acts on the surfaces of both tumor cells and nearby normal cells to disturb their normal growth control, perhaps by altering the mobilities of membrane receptors. If in addition some normal cells under such stimulus should be found to differentiate so as to express endogenous viral information and so generate more cell factor T_C, a positive feedback loop leading naturally to tumor growth would be shown to exist.

ACKNOWLEDGMENTS

We are indebted to Rex Risser, Nancy Hopkins, and Daniel Rifkin for a critical reading of the manuscript. P. H wishes to thank the John Simon Guggenheim Memorial Foundation for the award of a fellowship. This work was supported in part by the National Cancer Institute and by the US Atomic Energy Commission.

Literature Cited

1. Goldberg, N. D., O'Dea, R. F., Haddox, M. K. 1973. In *Recent Advances in Cyclic Nucleotide Research*, ed. P. Greengard, A. G. Robison, Vol. III. New York: Raven
2. Temin, H. M., Smith, G. L., Dulak, N. C. 1973. In *Control of Proliferation in Animal Cells*. Cold Spring Harbor, NY: Cold Spring Harbor Lab.
3. Hall, K., Van Wyk, J. J. 1973. In *Current Topics in Experimental Endocrinology*, ed. L. Martini, V. H. James. New York: Academic
4. Risser, R., Pollack, R. E. 1973. In *Control of Proliferation in Animal Cells*. Cold Spring Harbor, NY: Cold Spring Harbor Lab.
5. Martin, G. S. 1970. *Nature* 227:1021–23
6. Unkeless, J. C. et al 1973. *J. Exp. Med.* 137:85–111
7. Ossowski, L. et al 1973. *J. Exp. Med.* 137:112–26
8. Temin, H. M. 1967. *J. Biol. Chem.* 69:377–84
9. Anderson, W. B., Johnson, G. S., Pastan, I. 1973. *Proc. Nat. Acad. Sci. USA* 70:1055–59
10. Cunningham, D. D., Pardee, A. B. 1969. *Proc. Nat. Acad. Sci. USA* 64:1049–56
11. Edelman, G. M. 1973. In *Control of Proliferation in Animal Cells*. Cold Spring Harbor, NY: Cold Spring Harbor Lab.
12. Nicolson, G. L. 1973. In *Control of Proliferation in Animal Cells*. Cold Spring Harbor, NY: Cold Spring Harbor Lab.
13. Singer, S. J., Nicolson, G. L. 1972. *Science* 175:720–31
14. Caspar, D. L. D., Kirschner, D. A. 1971. *Nature New Biol.* 231:46–52
15. Tooze, J., Ed. 1973. *The Molecular Biology of Tumor Viruses*. Cold Spring Harbor, NY: Cold Spring Harbor Lab.
16. Oldfield, E., Singer, S. J. 1973. *Science* 180:982–83
17. Kreiner, P. W., Keirns, J. J., Bitensky, M. W. 1973. *Proc. Nat. Acad. Sci. USA* 70:1785–89
18. Berg, H. C. 1969. *Biochim. Biophys. Acta* 183:65–78
19. Bretscher, M. S. 1971. *J. Mol. Biol.* 58:775–81
20. Bretscher, M. S. 1971. *J. Mol. Biol.* 59:351–57
21. Phillips, D. R., Morrison, M. 1971. *FEBS Lett.* 18:95–97
22. Branton, D. 1966. *Proc. Nat. Acad. Sci. USA* 55:1048–56
23. Branton, D. 1969. *Ann. Rev. Plant Physiol.* 20:209–38
24. Pinto Da Silva, P., Branton, D. 1970. *J. Cell Biol.* 45:598–605
25. Engstrom, L. H. 1970. PhD thesis. Univ. Calif., Berkeley
26. Satir, P., Satir, B. 1973. In *Control of Proliferation in Animal Cells*. Cold Spring Harbor, NY: Cold Spring Harbor Lab.
27. Satir, B., Schooley, C., Satir, P. 1973. *J. Cell Biol.* 56:153–76
28. Frye, L. D., Edidin, M. 1970. *J. Cell Sci.* 7:319–35
29. Edidin, M., Weiss, A. 1972. *Proc. Nat. Acad. Sci. USA* 69:2456–59
30. Pollack, R., Green, H., Todaro, G. 1968. *Proc. Nat. Acad. Sci. USA* 60:126–33
31. Balk, S. D., Whitfield, J. F., Youdale, T., Braun, A. C. 1973. *Proc. Nat. Acad. Sci. USA* 70:675–79
32. Robison, A. G., Butcher, R. W., Sutherland, E. W. 1972. *Cyclic AMP*. New York: Academic
33. George, W. J., Polson, J. B., O'Toole, A. G., Goldberg, N. D. 1970. *Proc. Nat. Acad. Sci. USA* 66:398–403
34. Goldberg, N. D., Haddox, M. K., Hartle, D. K., Hadden, J. W. 1973. *Fifth International Congress of Pharmacology*. Basel: Karger
35. Pastan, I. et al 1973. In *Control of Proliferation in Animal Cells*. Cold Spring Harbor, NY: Cold Spring Harbor Lab.
36. Sheppard, J. R., Bannai, S. 1973. In *Control of Proliferation in Animal Cells*. Cold Spring Harbor, NY: Cold Spring Harbor Lab.
37. Sheppard, J. R. 1972. *Nature New Biol.* 236:14–16
38. Otten, J., Johnson, G. S., Pastan, I. 1971.

Biochem. Biophys. Res. Commun. 44:1192–98

39. Otten, J., Johnson, G. S., Pastan, I. 1972. *J. Biol. Chem.* 247:7082–87
40. Johnson, G. S., Pastan, I. 1972. *J. Nat. Cancer Inst.* 48:1377–87
41. Estensen, R. D. et al 1973. In *Control of Proliferation in Animal Cells.* Cold Spring Harbor, NY: Cold Spring Harbor Lab.
42. Holley, R. W. 1973. In *Control of Proliferation in Animal Cells.* Cold Spring Harbor, NY: Cold Spring Harbor Lab.
43. Holley, R. W., Kiernan, J. A. 1971. *CIBA Foundation Symposium, Growth Control in Cell Cultures,* ed. G. E. W. Wolstenholme, J. Knight, p. 3. London: Churchill Livingstone
44. Pierson, R. W. Jr., Temin, H. M. 1972. *J. Cell. Physiol.* 79:319–30
45. Dulak, N. C., Temin, H. M. 1973. *J. Cell. Physiol.* 81:153–60
46. Dulak, N. C., Temin, H. M. 1973. *J. Cell. Physiol.* 81:161–70
47. Salmon, W. D., Daughaday, W. H. 1957. *J. Lab. Clin. Med.* 49:825–36
48. Hintz, R. L., Clemmons, D. R., Underwood, L. E., Van Wyk, J. J. 1972. *Proc. Nat. Acad. Sci. USA* 69:2351–53
49. Cuatrecasas, P. 1972. *Proc. Nat. Acad. Sci. USA* 69:318–22
50. Coon, H. G. 1968. *J. Cell Biol.* 39:29a Abstr.
51. Gelb, L. D., Kohne, D. E., Martin, M. A. 1971. *J. Mol. Biol.* 57:129–45
52. Boyd, V. A. L., Butel, J. S. 1972. *J. Virol.* 10:399–409
53. Ozanne, B., Vogel, A., Sharp, P., Keller, W., Sambrook, J. 1973. *Lepetit Colloq. Biol. Med.* Vol. 4. In press
54. Gelb, L. D., Milstein, J. B., Martin, M. A., Aaronson, S. A. 1973. *Nature New Biol.* 244:76–78
55. Vogt, P. K. et al 1972. *M. D. Anderson Symposium on Fundamental Cancer Research, Houston, Texas.* Baltimore: Williams & Wilkins
56. Biquard, J.-M., Vigier, P. 1970. *C. R. Acad. Sci. D* 271:2430–33
57. Wyke, J. A., Linial, M. 1973. *Virology* 53:152–61
58. Kawai, S., Hanafusa, H. 1971. *Virology* 46:470–79
59. Biquard, J.-M., Vigier, P. 1972. *Virology* 47:444–55
60. Bader, J. P. 1972. *J. Virol.* 10:267–76
61. Martin, G. S. 1971. In *The Biology of Oncogenic Viruses,* ed. L. Silvestri, 320–25. Amsterdam: North Holland
62. Wyke, J. 1973. *Virology* 54:28–36
63. Friis, R. R., Toyoshima, K., Vogt, P. K. 1971. *Virology* 43:375–89
64. Ambrose, E. J., Dudgeon, J. A., Easty, D. M., Easty, G. C. 1961. *Exp. Cell Res.* 24:220–27
65. Aub, J. C., Tieslau, C., Lankester, A. 1963. *Proc. Nat. Acad. Sci. USA* 50:613–19
66. Burger, M. M. 1973. *Fed. Proc.* 32:91–101
67. Pollack, R. E., Burger, M. M. 1969. *Proc. Nat. Acad. Sci. USA* 62:1074–76
68. Inbar, M., Sachs, L. 1969. *Proc. Nat. Acad. Sci. USA* 63:1418–25
69. Arndt-Jovin, D. J., Berg, P. 1971. *J. Virol.* 8:716–21
70. Cline, M. J., Livingston, D. C. 1971. *Nature New Biol.* 232:155–56
71. Ozanne, B., Sambrook, J. 1971. *Nature New Biol.* 232:156–60
72. Nicolson, G. L. 1971. *Nature New Biol.* 233:244–46
73. Rosenblith, J. Z., Ukena, T. E., Yin, H. H., Berlin, R. D., Karnovsky, M. J. 1973. *Proc. Nat. Acad. Sci. USA* 70:1625–29
74. Edelman, G. M. et al 1972. *Proc. Nat. Acad. Sci. USA* 69:2580–84
75. Burger, M. M., Goldberg, A. R. 1967. *Proc. Nat. Acad. Sci. USA* 57:359–66
76. Taylor, R. B., Duffus, W. P. H., Raff, M. C., de Petris, S. 1971. *Nature New Biol.* 233:225–29
77. de Petris, S., Raff, M. C. 1972. *Eur. J. Immunol.* 2:523–35
78. Yahara, I., Edelman, G. M. 1972. *Proc. Nat. Acad. Sci. USA* 69:608–12
79. Edelman, G. M., Yahara, I., Wang, J. L. 1973. *Proc. Nat. Acad. Sci. USA* 70:1442–46
80. Yahara, I., Edelman, G. M. 1973. *Exp. Cell Res.* 81:143–55
81. Unanue, E. R., Perkins, W. D., Karnovsky, M. J. 1972. *J. Exp. Med.* 136:885–906
82. Gunther, G. R., Wang, J. L., Yahara, I., Cunningham, B. A., Edelman, G. M. 1973. *Proc. Nat. Acad. Sci. USA* 70:1012–16
83. Weisenberg, R. C., Borisy, G. C., Taylor, E. W. 1968. *Biochemistry* 7:4466–79
84. Behnke, O., Forer, A. 1967. *J. Cell Sci.* 2:169–92
85. Borisy, G. C., Olmsted, J. B., Klugman, R. A. 1972. *Proc. Nat. Acad. Sci. USA* 69:2890–94
86. Reich, E. 1973. In *Control of Proliferation in Animal Cells.* Cold Spring Harbor, NY: Cold Spring Harbor Lab.
87. Fischer, A. 1925. *Arch. Entwicklungsmech. Organismen* 104:210–22

88. Quigley, J. P., Ossowski, L., Reich, E. 1973. *J. Biol. Chem.* In press
89. Unkeless, J. C., Kellerman, G. M., Dano, K., Reich, E. 1973. *J. Biol. Chem.* In press
90. Ossowski, L., Quigley, J. P., Reich, E. 1973. *J. Biol. Chem.* In press
91. Ossowski, L., Quigley, J. P., Kellerman, G. M., Reich, E. 1973. *J. Exp. Med.* 138:1056–64

Copyright 1974. All rights reserved

INTRAVASCULAR COAGULATION IN LIVER DISEASE[1]

❖7124

M. Verstraete, J. Vermylen, and D. Collen
Laboratory of Blood Coagulation, University of Leuven, Belgium

Severe liver disease is frequently associated with a bleeding tendency and/or abnormalities in the laboratory tests of hemostasis. The latter comprise deficiencies of one or more of the coagulation factors II, V, VII, IX, X, XI, and XIII, thrombocytopenia and/or thrombocytopathia, and increased fibrinolytic activity in the plasma.

Several mechanisms are thought to be involved in the pathogenesis of these disturbances. The decreased levels of coagulation factors have generally been ascribed to impaired hepatic synthesis. However, production of defective molecules cannot be excluded. In fact, dysfibrinogenemia is relatively well documented in association with primary hepatoma and perhaps other forms of liver disturbances (1). Thrombocytopenia is rarely severe and appears to be related to excessive pooling of platelets in the congestive splenic sinusoids (2, 3), whereas the occasional functional alterations of platelets remain poorly understood (4–6).

Although fibrinolytic activity is rarely spontaneously increased in severe liver disease, it is well established that the fibrinolytic response to stimuli such as exercise (7) or injection of nicotinic acid (8, 9) or catecholamines (10) in cirrhosis of the liver is grossly exaggerated. It was therefore suggested that abnormal fibrinolysis in the cirrhotic patient may be due to a failure of the hepatic clearance of plasminogen activator. This hypothesis was also supported by the finding that the activator activity, generated in humans by streptokinase infusion, disappeared much less rapidly in a cirrhotic patient than in other subjects (11). Increased fibrinolytic activity in liver tissue (but probably not the hepatocytes) was demonstrated in extraction experiments (12) and by histochemical methods (13). The exaggerated fibrinolytic response in cirrhosis may be facilitated by decreased levels of circulating inhibitors. As the liver is the synthesis site of α_1-antitrypsin and α_2-macroglobulin (14), low levels of these protease inhibitors might be expected in patients with severe liver disease. Serum antiplasmin levels were found to be reduced in patients with

---

[1] Supported by Grant 1216 of the Fonds voor Wetenschappelijk Geneeskundig Onderzoek, Belgium.

decompensated liver insufficiency by some (15–19), but not others (8). In patients with less severe liver cirrhosis no good correlation was found between the extent of the liver damage and the α_1-antitrypsin level (20, 21).

Defective or abnormal synthesis and enhanced fibrinolytic activity cannot explain all facets of the defective hemostasis in certain patients with severe liver disease. An increased consumption of coagulation factors due to intravascular coagulation has been proposed as an alternative or complementary cause of the depletion of coagulation factors, at least in severe cases of liver cirrhosis and fulminant hepatic failure (22–24). This hypothesis was strengthened by the common clinical experience that the reduced levels of coagulation factors in patients with acute hepatic necrosis require more transfusions of concentrated coagulation factors or fresh plasma than anticipated (25–28). No evidence has yet been provided for the occurrence of disseminated in vivo coagulation in such liver diseases as obstructive jaundice and metastatic liver involvement.

SIMILARITY OF THE COAGULATION PROFILE IN DISSEMINATED INTRAVASCULAR COAGULATION AND IN SEVERE LIVER DISEASE

Characteristic laboratory findings of experimental disseminated intravascular coagulation include low levels of fibrinogen, prothrombin, and factor V, associated with an increase in the circulating level of fibrin(ogen) degradation products. Thrombocytopenia is frequently observed and is sometimes associated with hemolytic anemia and helmet red cells or schistocytes in the circulation. There is frequently a similarity between these complex coagulation and fibrinolytic disturbances and those observed in patients with severe liver insufficiency. It should be admitted that in low-grade cases not all abnormalities are present, which renders the appropriate diagnosis more difficult.

Mild thrombocytopenia, even when platelet production is still active, was observed in many patients with fulminant hepatic failure (28–31), severe liver cirrhosis (22–24, 32–34), and after homotransplantation of the liver (35). Fibrin degradation products were also often increased and the thrombin time prolonged even in the presence of normal fibrinogen levels. This prolongation of the thrombin time is generally due to circulating early breakdown products of fibrin or fibrinogen (fragments X and Y); high levels of gamma globulins could also prolong the thrombin time as would functionally abnormal fibrinogen. Because the circulating activator of fibrinolysis is rarely increased in baseline conditions, the elevated level of fibrin breakdown products is probably attributable to enhancement of local fibrinolytic activity in relation to intravascular fibrin deposition. During heparin administration there is often an improvement in the abnormal coagulation and fibrinolytic tests; this improvement with anticoagulation supports the concept of intravascular coagulation.

ACCELERATED CATABOLISM OF FIBRINOGEN, PROTHROMBIN, AND PLASMINOGEN IN LIVER DISEASE

Measurement of the turnover rate of radiolabeled components of the coagulation and fibrinolytic systems allows a quantitative determination of the relative importance of defective synthesis, intravascular coagulation, and fibrinolysis in the pathogenesis of the coagulation disturbances in liver disease. A decrease in the level of circulating fibrinogen, prothrombin, and plasminogen will only occur when the synthesis is markedly impaired or when the rate of catabolism is so excessively enhanced that maximal synthesis fails to compensate. Therefore, turnover studies of these factors could be particularly useful to study the kinetics of synthesis and breakdown and to differentiate their catabolic pathways in states of altered coagulability. Such studies require the availability of highly purified and biologically intact preparations of the protein marker under investigation. The feasibility of this approach has been illustrated for fibrinogen (36), prothrombin (37), and plasminogen (38).

It is generally accepted that under physiological conditions, fibrinogen (39–48) and prothrombin (49–51) are not or only to a minimal extent catabolized by consumption in the coagulation or fibrinolytic systems. This view has been recently challenged as circulating fibrinogen catabolites of high molecular weight were found under physiologic conditions (52). On the other hand, assay of circulating fibrinopeptide A levels indicated that at most 2–3% of the circulating fibrinogen is catabolized via thrombin proteolysis in normal subjects (53). Likewise the primary pathway of plasminogen under physiological conditions is not via formation of plasmin (54).

Tytgat et al (55) studied the metabolism of homologous human fibrinogen labeled with radioactive iodine in 50 patients with well documented cirrhosis of the liver. These patients were at various stages of the disease, but in a steady state with respect to fibrinogen metabolism. The mean plasma fibrinogen concentration of 250 mg ± 102 mg/100 ml in cirrhosis was not significantly lower than the control value of 284 ± 71 mg/100 ml in thirty five control subjects. The mean fibrinogen half-life of 2.99 ± 0.59 days was significantly shorter than the half-life of 4.14 ± 0.56 days in controls, whereas the mean fractional catabolic rate of 0.34 ± 0.09 of the plasma pool/day in cirrhosis was significantly higher than the control value of 0.24 ± 0.04. The mean quantity of fibrinogen catabolized daily, 2.66 ± 1.43 g or 39 ± 20 mg/kg in cirrhotics, was significantly higher than the corresponding mean control values of 1.94 ± 0.59 g or 28 ± 9 mg/kg. On the average the increased fibrinogen catabolism was counterbalanced by increased synthesis resulting in normal plasma fibrinogen levels.

During continuous heparin infusion in 10 cirrhotic patients the fibrinogen half-life was prolonged from 3.15 ± 0.69 to 4.59 ± 0.79 days. Inhibition of the fibrinolytic system by peroral administration of tranexamic acid (Cyklokapron®) did not influence the fibrinogen half-life consistently. It was therefore concluded that the accelerated fibrinogen consumption was mainly due to continuous intravascular coagulation.

These findings in a large group of patients confirm and extend the earlier results of other investigators who studied a few patients with cirrhosis and applied the same technique for labeling fibrinogen (42, 45), or used in vivo incorporation of labeled selenomethionine (56). The turnover rate of labeled fibrinogen was also found to be considerably increased in acute hepatic necrosis (30) and acute viral hepatitis (57).

The plasma levels of prothrombin, the zymogen which is finally converted in the coagulation mechanism, and of plasminogen, the precursor of the fibrinolytic enzyme plasmin, are frequently subnormal in cirrhosis of the liver. This may be the result of either diminished hepatic synthesis, or excessive consumption, or both.

Turnover studies of labeled prothrombin, prepared by the method of Swart (58), have been performed by Collen et al (59) in six patients with cirrhosis of the liver to evaluate the relative importance of defective synthesis and in vivo coagulation in the pathogenesis of the hypoprothrombinemia. Compared to normals, the cirrhotic patients showed a significantly increased fractional catabolic rate and a decreased synthetic rate. Heparin administration in three patients resulted in a normalization of the prothrombin half-life. However, because the plasma prothrombin level was correlated with the synthetic rate but not with the fractional catabolic rate, it was concluded that the hypoprothrombinemia was mainly caused by the impaired synthetic ability of the diseased liver.

This shortening of the half-life of labeled prothrombin in cirrhosis of the liver corresponds with the findings of Didisheim et al (60) who administered a prothrombin-rich fraction to a cirrhotic patient and obtained a half-life of approximately 40 hr, while Tullis et al (61) obtained values of less than 24 hr in apparently similar patients.

Turnover studies of labeled plasminogen (59), performed in four patients with cirrhosis of the liver, revealed an increased fractional catabolic rate and a decreased synthesis rate of plasminogen; heparin administration in two patients resulted in a normalization of the turnover rate. As was observed for prothrombin, the plasma plasminogen level was correlated with the synthetic rate but not with the fractional catabolic rate, indicating that the hypoplasminogenemia was also mainly the result of decreased synthesis.

Two hypotheses can be presented to explain the accelerated plasminogen catabolism in cirrhosis: primary or secondary activation of the fibrinolytic system or absorption of plasminogen onto in vivo evolving fibrin. The absence of overt signs of fibrinolytic activity does not necessarily invalidate the hypothesis of primary or secondary activation of the fibrinolytic system. Indeed the dynamic study of the plasminogen turnover should be much more sensitive in detecting accelerated consumption than the static picture of circulating levels of plasminogen, plasminogen activator, or fibrin(ogen) degradation products. The finding that heparin administration induced a prolongation of the plasminogen half-life in two patients with cirrhosis (59) becomes a less stringent argument in favor of enhanced fibrinolysis secondary to intravascular coagulation in view of the experiments of Takeda et al (62), which show that very large doses of heparin produce a marked inhibition of plasmin generation by urokinase in dogs.

If, and to what extent, there is in vivo absorption of plasminogen onto fibrin with subsequent enhanced clearing of the complex, has not yet been evaluated experimentally.

FINDINGS SUGGESTIVE OF INTRAVASCULAR COAGULATION IN EXPERIMENTAL ANIMALS WITH INDUCED LIVER DISORDERS

Estimation of the relative importance of abnormalities in the liver cell system (intrahepatic factors) and of the disturbances in hepatoportal blood flow (extrahepatic factors) in the pathogenesis of the accelerated turnover of coagulation factors in cirrhosis cannot be investigated in man. Our laboratory therefore attempted to differentiate the influence of both factors in dogs (63). Primary damage of the liver cell system was induced by prolonged liver intoxication with carbon tetrachloride (CCl_4) and ethanol. Primary abnormalities of the portal blood flow were induced by progressive ligation and finally sectioning of the portal vein. Prolonged administration of CCl_4-ethanol induced successive waves of hepatocellular necrosis with subsequent fibrotic dissection and nodular disorganization. These lesions were of variable severity and apparently not associated with portal hypertension. A significantly shortened fibrinogen survival in association with a general trend towards prolongation and occasionally normalization of the fibrinogen life span during heparinization was observed. This finding strongly supports the hypothesis of increased fibrinogen catabolism due to low-grade, possibly regional in vivo coagulation in these animals.

Longstanding interruption of hepatoportal blood flow by gradual occlusion and transsection of the portal vein in dogs did not by itself induce apparent changes in fibrinogen metabolism in spite of the congestive splenomegaly and extensive collateral circulation. The latter experiments therefore do not support the hypothesis that disturbance in portal hemodynamics with splenomegaly and collateral circulation are of primary importance in the pathogenesis of the intravascular consumption syndrome in cirrhosis.

Parenteral administration of galactosamine in rats and rabbits induced histological changes and laboratory findings in liver resembling those that can be found in acute hepatitis of humans. In this experimental model hypercoagulability, as determined by the thrombin generation test, was found in the first 12 hr and was followed by a decrease of most clotting factors and platelets, suggesting intravascular coagulation. These changes could be partly prevented by heparin (64).

Also, during liver homotransplantation in the pig, there is an anhepatic phase with coagulation changes closely resembling a consumption coagulopathy (65–67).

PATHOGENESIS OF INTRAVASCULAR COAGULATION IN SEVERE LIVER DISORDERS

A reasonable explanation for the occurrence of intravascular coagulation in patients with severe liver insufficiency is that necrotic hepatocytes are the initial trigger. In addition, activated coagulation factors are less opposed because of defective clearing in the reticuloendothelial system and lowered levels of antithrombin in liver disease. These different elements are now analyzed in some detail.

Consumption coagulopathy is often seen in states where blood comes into contact with damaged cells; tissue thromboplastin-like material is released in response to cellular injury, with consequent activation of the coagulation process and thrombin-induced aggregation of platelets. The original stimulus to intravascular coagulation in liver disease probably comes from the necrotic hepatocytes. The clearance of procoagulants thus generated may be diminished because of liver insufficiency (68). Furthermore, in cirrhotic patients the expanded collateral circulation and congested spleen represent a tremendous increase in endothelial surface. Sluggish circulation in this collateral vascular bed and in dilated splenic sinusoids, where a high number of platelets are trapped, might well create (hypoxic?) alterations of the endothelial cells and predispose to local clotting. This hypothesis is supported by the finding of a shortened fibrinogen half-life in patients with extrahepatic portal hypertension, splenomegaly, and extensive collateral circulation but with normal liver function and architecture (55). It has also been reported that in congestive splenomegaly coagulation abnormalities (e.g. thrombocytopenia and low factor V levels), presumably of the consumption type, can be more or less completely reversed by splenectomy (69, 70). This finding is at variance with the normal fibrinogen turnover found in dogs with portal vein occlusion. However, some similarity can be seen between the extensive collateral circulation in cirrhosis and a giant hemangioma, which is also frequently associated with intravascular coagulation (33, 71). As overt hemolysis of different degrees is often a feature in liver cirrhosis (72), release of phospholipid and adenosine diphosphate from the red cells could also trigger in vivo coagulation and platelet aggregation. Thus in patients with severe liver insufficiency a variety of stimuli for the initiation of in vivo coagulation is present.

Since intravascular fibrin thrombi in the liver sinusoids were observed in toxic hepatic necrosis (73) and can cause further liver damage, and since fragmentation of red cells (so-called microangiopathic hemolytic anemia) may cause further release of thromboplastic material, a vicious circle may result which can perpetuate intravascular coagulation.

Activated coagulation factors are normally rapidly removed from the blood stream due to the clearing function of the liver. This property was convincingly demonstrated in animals by Wessler et al (68) and Deykin (74). When thrombin-free normal human serum was injected through the marginal vein of a rabbit, a hypercoagulable state was produced, resulting in rapid clot formation in an isolated venous segment (68). The infusion of thrombin-free normal human serum through the portal vein accelerated the rate of attenuation of the hypercoagulable response. In contrast, occlusion of the hepatic circulation after the injection of serum prolonged the hypercoagulable state, and widespread thrombosis occurred in areas of vascular stasis. Finally it was shown that the thrombogenic activity of normal human serum was cleared by perfusion through isolated rabbit liver (74). Furthermore, the liver possesses a remarkable capacity to distinguish between the nonactivated and activated clotting factors, only the latter being very efficiently removed from the bloodstream (75).

When minute amounts of thrombin are generated, the action of this enzyme can also more readily develop when the antithrombin level is reduced, as is often observed in patients with liver insufficiency.

CONCLUSION

It can be concluded that the biological integrity of the liver cell system appears of utmost importance in preventing in vivo activation of the coagulation system. Not only are the liver cells the principal site of synthesis of the bulk of coagulation and fibrinolytic proteins, but they are also invaluable in equilibrating clot-promoting forces.

It is presently well documented that the levels of certain coagulation factors are decreased in severe liver insufficiency. Furthermore, the catabolism of fibrinogen, prothrombin, and plasminogen is enhanced in liver cirrhosis. The consistent normalization of the fibrinogen half-life by heparin administration strongly suggests that continuous low-grade in vivo coagulation is responsible for the increased catabolism; usually the increased metabolism is counterbalanced by an enhanced synthesis resulting in normal plasma fibrinogen levels. On the contrary, the decreased levels of prothrombin and plasminogen frequently encountered in this condition are mainly due to an insufficient synthesis and only to a minor degree to enhanced catabolism.

Although the present data indicate that low-grade intravascular coagulation is a frequent complication of severe liver disease, overt consumption coagulopathies requiring therapeutic anticoagulation are rare.

Literature Cited

1. Verhaeghe, R., Van Damme, B., Molla, A., Vermylen, J. 1973. *Scand. J. Haematol.* 9:451–58
2. Aster, R. H. 1966. *J. Clin. Invest.* 45:645–57
3. Penny, R., Rozenberg, M. C., Firkin, B. G. 1966. *Blood* 27:1–16
4. Mandel, E. E., Lazerson, J. 1961. *N. Engl. J. Med.* 265:56–61
5. Cortet, P., Klepping, C., Devant, J. 1964. *Arch. Fr. Mal. Appar. Dig.* 53:1041–45
6. Thomas, D. P., Ream, V. J., Stuart, R. K. 1967. *N. Engl. J. Med.* 276:1344–48
7. Das, P. C., Cash, J. D. 1969. *Brit. J. Haematol.* 17:431–43
8. Fletcher, A. P., Biederman, O., Moore, D., Alkjaersig, N., Sherry, S. 1964. *J. Clin. Invest.* 43:681–95
9. Tytgat, G. N., Collen, D. J., De Vreker, R. A. 1968. *Nouv. Rev. Fr. Hematol.* 8:123–31
10. Kwaan, H. C., McFadzean, A. J., Cook, J. 1956. *Lancet* 1:132
11. Amery, A. 1969. *Activation of the human plasma fibrinolytic system by streptokinase.* PhD thesis. Univ. Leuven, Belgium. 141 pp.
12. Astrup, T., Rasmussen, J., Amery, A., Poulsen, H. E. 1960. *Nature* 185:619–20
13. Denk, H., Schnack, H., Deutsch, E. 1970. *Acta Hepato Splenol.* 17:221–28
14. Schultze, H. E., Heremans, J. F. 1966. *Molecular Biology of Human Proteins.* Vol. 1. Amsterdam: Elsevier. 688 pp.
15. Beaumont, J., Beaumont, V., Domart, A. 1956. *Rev. Fr. Etud. Clin. Biol.* 1:667–73
16. Purcell, G., Phillips, L. L. 1963. *Surg. Gynecol. Obstet.* 117:139–44
17. O'Connell, R. A., Grossi, C. E., Rousselot, L. M. 1964. *Lancet* 2:990–91
18. Tytgat, G., Collen, D., De Vreker, R. A., Verstraete, M. 1968. *Acta Haematol.* 40:265–74

19. Ratnoff, O. D. 1969. *Diseases of the Liver,* ed. L. Schiff. Philadelphia: Lippencott. 1102 pp.
20. Sharp, H. L., Bridges, R. A., Krivit, W., Freier, E. F. 1969. *J. Lab. Clin. Med.* 73:934–39
21. Lieberman, J., Mittman, C. 1970. *Ann. Intern. Med.* 73:9–13
22. Bergström, K., Blombäck, B., Kleen, G. 1960. *Acta Med. Scand.* 168:291–305
23. Zetterqvist, E., von Francken, I. 1963. *Acta Med. Scand.* 173:753–60
24. Johansson, S. A. 1964. *Acta Med. Scand.* 175:177–83
25. Spector, I., Corn, M., Ticktin, H. E. 1966. *N. Engl. J. Med.* 275:1032–37
26. Jones, E. A., Clain, D., Clink, H. M., MacGillivray, M., Sherlock, S. 1967. *Lancet* 11:169–72
27. Tytgat, G., Piessens, J., Collen, D., De Groote, J. 1968. *Digestion* 1:257–66
28. Ménaché, D. et al 1968. *Prob. Réanim.* 5:499–553
29. Niléhn, J. E., Nilsson, I. M., Aronsen, K. F., Erricsson, B. 1967. *Acta Chir. Scand.* 133:189–95
30. Rake, M. O., Pannell, G., Flute, P. T., Williams, R. 1970. *Lancet* 1:533–37
31. Dennis, L. H., Reisberg, B. E., Crosbie, J., Crozier, D., Conrad, M. E. 1969. *Brit. J. Haematol.* 17:455–62
32. Lewis, J. H., Doyle, A. P. 1964. *J. Am. Med. Assoc.* 188:56–63
33. Verstraete, M., Vermylen, C., Vermylen, J. 1965. *Am. J. Med.* 38:899–908
34. Gajewski, J., Gurewich, V., Mobarhan, S., Hutchinson, B. S. 1971. *Hemostatic Abnormalities in Liver Cirrhosis.* Presented at Int. Congr. Thromb. Haemostasis, Oslo, Norway
35. Flute, P. T., Rake, M. O., Williams, R., Seamar, M. J., Calne, R. Y. 1969. *Brit. Med. J.* 3:20–23
36. Tytgat, G., Collen, D., Vermylen, J. 1972. *Brit. J. Haematol.* 22:701–17
37. Shapiro, S. S., Martinez, J. 1971. *The Use of* ^{125}*I-Prothrombin in the Study of States of Altered Coagulability.* Presented at Int. Congr. Thromb. Haemostasis, Oslo, Norway
38. Collen, D., Vermylen, J. 1973. *Thromb. Res.* 2:239–49
39. Christensen, L. K. 1958. *Acta Med. Scand.* 162:407–14
40. Hammond, D. J. S., Verel, D. 1959. *Brit. J. Haematol.* 5:431–38
41. Adelson, E., Rheingold, J. J., Parker, O., Buenaventura, A., Crosby, W. H. 1961. *Blood* 17:267–81
42. Amris, A., Amris, C. J. 1964. *Thromb. Diath. Haemorrh.* 2:404–22
43. McFarlane, A. S., Todd, D., Cromwell, S. 1964. *Clin. Sci.* 26:415–20
44. Hart, H. Ch. 1966. *Thromb. Diath. Haemorrh. Suppl.* 17:121–31
45. Blombäck, B., Carlson, L. A., Franzén, S., Zetterqvist, E. 1966. *Acta Med. Scand.* 179:557–74
46. Takeda, Y. 1966. *J. Clin. Invest.* 45:103–11
47. Izak, G., Calewski, K., Eyal, Z. 1967. *Thromb. Diath. Haemorrh.* 18:544–51
48. Collen, D., Tytgat, G., Claeys, H., Piessens, R. 1972. *Brit. J. Haematol.* 22:681–700
49. Shapiro, S. S., Martinez, J. 1969. *J. Clin. Invest.* 48:1292–98
50. Takeda, Y. 1972. *Thromb. Diath. Haemorrh.* 27:472–89
51. Rouvier, J., Collen, D., Swart, A. C. W., Verstraete, M. Unpublished
52. Mosesson, M. W. 1973. *Thromb. Res.* 2:185–200
53. Nossel, H. L., Canfield, R. E., Butler, V. P. 1973. *Plasma Fibrinopeptide A Concentration as an Index of Intravascular Coagulation.* Presented at Int. Congr. Thromb. Haemostasis, Oslo, Norway
54. Collen, D., Tytgat, G., Claeys, H., Verstraete, M., Wallen, P. 1972. *J. Clin. Invest.* 51:1310–18
55. Tytgat, G. N., Collen, D., Verstraete, M. 1971. *J. Clin. Invest.* 50:1690–1701
56. Brodsky, I., Siegel, N. H., Benham Kahn, S., Ross, E. M., Petkov, G. 1970. *Brit. J. Haematol.* 18:341–55
57. Dechavanne, M., Bouletreau, P., Petit, P 1971. *Presse Med.* 79:2485–86
58. Swart, A. C. W. 1971. *Studies on the purification and separation of blood coagulation factors II, VII, IX and X.* PhD thesis. Univ. Leiden, Netherlands
59. Collen, D., Rouvier, J., Verstraete, M. 1972. *Clin. Res.* 20:483 (Abstr.)
60. Didisheim, P., Loeb, J., Blatrix, C., Soulier, J. P. 1959. *J. Lab. Clin. Med.* 53:322–30
61. Tullis, J. L., Melin, M., Jurigian, P. 1965. *N. Engl. J. Med.* 273:667–74
62. Takeda, Y., Parkhill, T. R., Nakabayashi, M. 1972. *J. Clin. Invest.* 51:2678–85
63. Tytgat, G. N., Collen, D., Oei, L. S., VanDamme, B. 1972. *Thromb. Diath. Haemorrh.* 27:319–36
64. Köstering, H. 1973. *Disseminated Intravascular Coagulation in Experimental Hepatitis Due to Galactosamine.* Presented at Int. Congr. Thromb. Haemostasis, Vienna, Austria
65. Von Kaulla, K. N., Kaye, H., von Kaulla, E., Marchioro, T. L., Starzl, T. 1966. *Arch. Surg.* 92:71–79

66. Moriau, M., Kestens, P. J., Masure, R. 1969. *Pathol. Eur.* 4:172–82
67. Coccheri, S., Faenza, A., Alessandri, M., Palareti, G., De Rosa, V. 1971. *Coagulation Disorders During and After Liver Homotransplantation in the Pig.* Presented at Int. Congr. Thromb. Haemostasis, Oslo, Norway
68. Wessler, S. Yin, E. T., Gaston, L. W., Nicol, I. 1967. *Thromb. Diath. Haemorrh.* 18:12–23
69. Vergoz, D., Levy, V. G., Najman, A., Caroli, J. 1967. *Rev. Fr. Etud. Clin. Biol.* 12:725–31
70. Fisher, S. 1968. *J. Am. Med. Assoc.* 205:111–12
71. Blix, S., Aas, K. 1961. *Acta Med. Scand.* 169:63–70
72. Kimber, C. J., Deller, D. J., Ibbotson, R. N., Lander, H. 1965. *Quart. J. Med.* 34:33–64
73. Popper, H., Franklin, M. 1948. *Arch. Pathol.* 46:338–76
74. Deykin, D. 1966. *J. Clin. Invest.* 45:256–63
75. Deykin, D., Cochios, F., Decamp, G., Lopez, A. 1968. *Am. J. Physiol.* 214:414–19

REPRINTS

The conspicuous number aligned in the margin with the title of each article in this volume is a key for use in ordering reprints.

Available reprints are priced at the uniform rate of $1 each postpaid. Payment must accompany orders less than $10. A discount of 20% will be given on orders of 20 or more. For orders of 200 or more, any Annual Reviews article will be specially printed.

The sale of reprints of articles published in the Reviews has been expanded in the belief that reprints as individual copies, as sets covering stated topics, and in quantity for classroom use will have a special appeal to students and teachers.

CUMULATIVE INDEXES

CONTRIBUTING AUTHORS VOLUMES 21-25

A

Abelmann, W. H., 24:145
Arnone, A., 25:123
Atkinson, A. J., 25:99

B

Bagdade, J. D., 21:219
Baldessarini, R. J., 23:343
Barker, W. H. Jr., 25:75
Barlow, G. H., 24:335
Barnes, A. C., 22:133
Bausher, J. C., 23:39
Becker, M. A., 25:15
Bellingham, A. J., 22:221
Bernard, J., 25:39
Bertino, J. R., 21:433
Biggar, W. D., 24:135
Bitensky, M. W., 23:263
Blumberg, B. S., 23:161
Bodey, G. P., 21:105
Bothwell, T. H., 21:145
Bouhuys, A., 24:111
Brady, R. O., 21:317
Brain, M. C., 21:133
Breslow, J. L., 24:315
Brewer, G. J., 25:29
Brummer, D. L., 25:115
Brusilow, S. W., 21:99
Buckner, C. D., 25:179
Burch, G. E., 22:327
Burchenal, J. H., 23:77
Burke, J. F., 24:289

C

Canterbury, J. M., 24:217
Capizzi, R. L., 21:433
Carpenter, C. B., 21:1
Charlton, R. W., 21:145
Childs, B., 23:373
Coburn, R. F., 24:241
Cohn, K. E., 21:47
Collen, D., 25:447
Collinsworth, K. A., 25:143
Coombs, J., 23:149
Cooperband, S. R., 22:185
Cotzias, G. C., 22:305
Creger, W. P., 24:75
Cuatrecasas, P., 24:233

D

Dao, T. L., 23:1
Davison, R., 25:99
De Clercq, E., 21:17
Deftos, L. J., 25:323
DeGraff, A. C., 24:111
DeLuca, H. F., 25:349
DePasquale, N. P., 22:327
Desbuquois, B., 24:217
Dixon, F. J., 25:83
Dowling, M. D. Jr., 23:77
Dreizen, P., 22:365
Dripps, R. D., 22:293
Düby, S., 22:305
Dupont, H. L., 24:103
Dupré, J., 21:299

E

Edgington, T. S., 25:303
Ehrenkrantz, N. J., 25:9
Eriksson, M., 24:29

F

Farber, E. M., 21:59
Fefer, A., 25:179
Fernstrom, J. D., 25:1
Field, J. B., 24:309
Finch, C. A., 22:221
Formal, S. B., 24:103
Fox, A. C., 22:355
Fredrickson, D. S., 24:315
Freedman, D. X., 24:153
Friesen, H. G., 24:251
Fudenberg, H. H., 22:145; 24:175

G

Gangarosa, E. J., 25:75
Gershon, S., 23:439
Gill, J. R. Jr., 21:269
Ginos, J., 22:305
Glassman, E., 23:229
Glenner, G. G., 25:131
Glucksberg, H., 25:179
Gold, P., 22:85
Goldstein, G., 22:119
Good, R. A., 24:135
Gorman, R. E., 23:263
Gottlieb, L. S., 25:411
Gray, G. M., 22:391
Greenberg, P. L., 25:269
Guenter, D. A., 22:283

H

Hammarsten, J. F., 22:283
Handschumacher, R. E., 21:433
Harker, L. A., 25:383
Harrison, D. C., 25:143
Hellström, I., 23:19
Hellström, K. E., 23:19
Henkind, P., 22:95
Hersh, E. M., 21:105
Hinman, F., 24:83
Hirschhorn, K., 24:67
Holick, M. F., 25:349
Holtzman, N. A., 21:335
Hornick, R., 24:103
Horton, E. S., 22:235
Hossfeld, D. K., 21:379
Hough, P. V. C., 25:431
Huehns, E. R., 21:157
Hurvitz, A. I., 23:191
Hwang, P., 24:251

I

Ishizaka, K., 21:187
Israel, C. W., 22:103

J

Jacquillat, C., 25:39
Janoff, A., 23:177
Johnson, J. W. C., 24:165
Johnson, M. T., 25:251
Jones, S. R., 25:251

K

Kalkhoff, R. K., 23:429
Kaltman, A. J., 22:343
Karpatkin, S., 23:101
Kayden, H. J., 23:285
Keele, C. A., 21:67
Kelly, J. F., 25:53
Kessler, R. H., 23:365
Kessler, S., 24:89
King, L. J., 21:367

Klebanoff, S. J., 22:39
Kleeman, C. R., 21:259
Klerman, G. L., 25: 199
Koenig, M. G., 22:25
Koffler, D., 25:149
Kolff, W. J., 23:321
Kwaan, H. C., 24:335

L

La Du, B. N., 23:453
Lane, J. M., 22:251
Lasseter, K. C., 25:69
Leader, R. W., 23:191
Legator, M. S., 23:413
Lenfant, C., 22:221
Lerner, K. G., 25:179
Levey, G. S., 25:69
Levin, A. S., 24:175
Levin, N. W., 24:365
Lewy, P. R., 24:365
Littlefield, J. W., 23:57
London, W. T., 23:161
Louria, D. B., 25:219
Lowenstein, J., 23:333
Luby, J. P., 25:251

M

McCarty, D. J., 21:357; 25:279
McConnell, R. B., 25:165
McCullough, J. L., 24:345
McGee, Z. A., 22:25
McGuigan, J. E., 25:233
Menguy, R., 23:297, 313
Merigan, T. C., 21:17
Merimee, T. J., 25:137
Millar, J. D., 22:251
Millman, I., 23:161
Mills, I. H., 21:75
Mills, J. A., 22:185
Milunsky, A., 23:57
Mitchell, J. H., 25:369
Moos, R. H., 24:39
Moss, G. S., 23:201
Murphy, D. L., 24:209

N

Neer, R., 25:323
Neff, J. M., 22:251
Neill, C. A., 24:61
Neiman, P., 25:179
Nyhan, W. L., 24:41

O

Odell, G. B., 24:165

P

Palmer, R. F., 25:69
Papavasiliou, P. S., 22:305
Park, B. H., 24:135
Patterson, R., 25:53
Perkoff, G. T., 22:125
Perry, S., 22:171
Pink, R., 22:145
Pollack, R. E., 25:431
Porte, D. Jr., 21:219
Potolsky, A., 24:75

Q

Quintanilla, A., 24:365

R

Rabinowitz, D., 21:241
Rabinowitz, M., 24:245
Raisfeld, I. H., 24:385
Ramot, B., 22:19
Reiss, E., 24:217
Remington, J. S., 21:201
Robertson, G. L., 25:315
Ross, G. T., 24:295
Rubenstein, A. H., 22:1

S

Salans, L. B., 22:235
Sandberg, A. A., 21:379
Sanford, J. P., 25:401
Schaffner, W., 22:25
Schildkraut, J. J., 25:333
Schrier, S. L., 25:269
Seegmiller, J. E., 25:15
Seligman, M. E. P., 23: 407
Selzer, A., 21:47
Senay, E. C., 24:153
Sharp, G. W. G., 24 19
Shaw, M. W., 21:409
Sims, E. A. H., 22:235
Sinclair, J. C., 23:129
Smith, J. L., 22:103
Smith, R. T., 23:39
Sokal, J. E., 24:281
Sokoloff, L., 24:271
Spencer, F. C., 23:229
Spitler, L., 24:175

Stamatoyannopoulos, G., 22:221
Stead, E. A. Jr., 22:273
Steck, A., 22:305
Steiner, D. F., 22:1
Storb, R., 25:179
Stutzman, L., 24:325
Sutnick, A. I., 23:161

T

Tan, C. T. C., 23:77
Tantum, K. R., 22:293
Terry, W. D., 25:131
Thomas, E. D., 25:179
Thomas, L., 21:179
Tomasi, T. B. Jr., 21:281
Trey, C., 25:411
Tschopp, T. B., 24:1

U

Unger, R. H., 24:303
Utiger, R. D., 25:289

V

Vaitukaitis, J., 24:295
Valentine, W. N., 23:93
Ventura, A. K., 25:9
Vermylen, J., 25:447
Verstraete, M., 25:447

W

Wang, A.-C., 22:145
Weil, M., 25:39
Weinstein, G. D., 24:345
Welch, M. H., 22:283
Wender, P. H., 23:355
Wilber, J. F., 24:353
Wildenthal, K., 25:369
Wilhelm, D. L., 22:63
Wilson, C. B., 25:83
Winick, M., 23:149
Woodrow, J. C., 25:165

Y

Yaffe, S. J., 24:29

Z

Zackheim, H. S., 21:59
Zak, R., 23:245
Zimmerman, T. S., 25:303
Zucker, M. B., 24:1

CHAPTER TITLES VOLUMES 21-25

CARDIOLOGY

Treatment of Ventricular Extrasystoles and Tachyarrhythmias in Acute Myocardial Infarction	A. Selzer, K. E. Cohn	21:47-58
Papillary Muscle Dysfunction in Coronary (Ischemic) Heart Disease	N. P. DePasquale, G. E. Burch	22:327-42
Late Complications of Heart Valve Replacement	A. J. Kaltman	22:343-54
Abnormalities in Cardiac Contractility Associated with Localized Myocardial Damage	A. C. Fox	22:355-64
Structure and Function of the Myofibrillar Contractile Proteins	P. Dreizen	22:365-90
Pulmonary Involvement in Hypovolemic Shock	G. S. Moss	23:201-28
Surgical Procedures for Coronary Artery Disease	F. C. Spencer, E. Glassman	23:229-44
Biochemical and Cellular Changes in Cardiac Hypertrophy	M. Rabinowitz, R. Zak	23:245-62
Genetics of Congenital Heart Disease	C. A. Neill	24:61-67
Viral Myocarditis and Its Sequelae	W. H. Abelmann	24:145-52
Primary Hyperlipoproteinemia in Infants	D. S. Fredrickson, J. L. Breslow	24:315-24
Sulfonylureas and the Heart	G. S. Levey, K. C. Lasseter, R. F. Palmer	25:69-74
Diphenylhydantoin as an Antiarrhythmic Drug	A. J. Atkinson, R. Davison	25:99-113
Antiarrhythmic Actions of Lidocaine	D. C. Harrison, K. A. Collinsworth	25:143-48
Static (Isometric) Exercise and the Heart: Physiological and Clinical Considerations	J. H. Mitchell, K. Wildenthal	25:369-81

DERMATOLOGY

Topical Antimetabolites	H. S. Zackheim, E. M. Farber	21:59-66
Chemical Causes of Pain and Itch	C. A. Keele	21:67-74
Cytokinetics in Diseases of Epidermal Hyperplasia	G. D. Weinstein, J. L. McCullough	24:345-52

ENDOCRINOLOGY

Human Insulin Secretion: An Integrated Approach	D. Porte Jr., J. D. Bagdade	21:219-40
Some Endocrine and Metabolic Aspects of Obesity	D. Rabinowitz	21:241-58
Hypo-Osmolar Syndromes Secondary to Impaired Water Excretion	C. R. Kleeman	21:259-68
Edema	J. R. Gill Jr.	21:269-80
Proinsulin	A. H. Rubenstein, D. F. Steiner	22:1-18
Chemical Mediation of Hormone Action	M. W. Bitensky, R. E. Gorman	23:263-84
Abetalipoproteinemia	H. J. Kayden	23:285-96
Blood Levels of Parathyroid Hormone in Disorders of Calcium Metabolism	E. Reiss, J. M. Canterbury	24:217-32
Insulin Receptors	B. Desbuquois, P. Cuatrecasas	24:233-40
Human Prolactin	H. Friesen, P. Hwang	24:251-70
Recent Advances in Evaluation of Gonadotropic Hormones	J. L. Vaitukaitis, G. T. Ross	24:295-302
Normal and Abnormal Alpha Cell Function	R. H. Unger	24:303-8
Thyrotropin Releasing Hormone: Secretion and Actions	J. F. Wilber	24:353-64
Isolated Growth Hormone Deficiency and Related Disorders	T. J. Merimee	25:137-42
Serum Triiodothyronine in Man	R. D. Utiger	25:289-302
Vasopressin in Osmotic Regulation in Man	G. L. Robertson	25:315-22
Vitamin D Metabolism	M. F. Holick, H. F. DeLuca	25:349-67

GASTROENTEROLOGY

Structure and Function of Mucosal Antibodies	T. B. Tomasi Jr.	21:281-98
Regulation of the Secretions of the Pancreas	J. Dupré	21:299-316
Malabsorption Due to Lymphomatous Disease	B. Ramot	22:19-24
Intestinal Digestion and Maldigestion of Dietary Carbohydrates	G. M. Gray	22:391-404
Acute Gastric Mucosal Bleeding	R. Menguy	23:297-312
Choice of Operations in the Management of Symptomatic Sliding Hiatal Hernia	R. Menguy	23:313-20
Action of Cholera Toxin on Fluid and Electrolyte Movement in the Small Intestine	G. W. G. Sharp	24:19-28
Enterotoxic Diarrheal Syndromes	S. B. Formal, H. L. Dupont, R. E. Hornick	24:103-10
Extraction of Insulin by Liver	J. B. Field	24:309-14
Role of Gastrin in Hypersecretory Disorders in Man	J. E. McGuigan	25:233-49

HEMATOLOGY

Leukocytic Mechanisms in Inflammation	E. M Hersh, G. P. Bodey	21:105-32
Microangiopathic Hemolytic Anemia	M. C Brain	21:133-44
Absorption of Iron	T. H. Bothwell, R. W. Charlton	21:145-56
Diseases Due to Abnormalities of Hemoglobin Structure	E. R. Huehns	21:157-78
Proliferation of Myeloid Cells	S. Perry	22:171-84
Lymphocyte Physiology	J. A. Mills, S. R. Cooperband	22:185-220
Abnormal Hemoglobins with High and Low Oxygen Affinity	G. Stamatoyannopoulos, A. J. Bellingham, C. Lenfant, C. A. Finch	22:221-34
Treatment of Acute Lymphoblastic Leukemia	J. H. Burchenal, M. D. Dowling Jr. C. T. C. Tan	23:77-92
Red Cell Enzyme Deficiencies as a Cause of Hemolytic Disorders	W. N. Valentine	23:93-100
Human Platelet Senescence	S. Karpatkin	23:101-28
Platelet Function Tests in Disease	T. B. Tschopp, M. B. Zucker	24:1-18
Radiation and Drug Therapies, and Leukemia	A. Potolsky, W. P. Creger	24:75-82
Current Concepts in the Treatment of Chronic Myelocytic Leukemia	J. E Sokal	24:281-88
Nature and Biological Activities of Degradation Products of Fibrinogen and Fibrin	H. C. Kwaan, G. H. Barlow	24:335-44
2, 3-DPG and Erythrocyte Oxygen Affinity	G. J. Brewer	25:29-38
Treatment of Acute Granulocytic Leukemias	J. Bernard, M. Weil, C. Jacquillat	25:39-51
Mechanism of Action of Hemoglobin	A. Arnone	25:123-30
Immuno-Prevention of Rh Hemolytic Disease of the Newborn	R. E. McConnell, J. C. Woodrow	25:165-78
Marrow Transplantation for Aplastic Anemia and Acute Leukemia	P. Neiman, E. D. Thomas, C. D. Buckner, R. Storb, A. Fefer, H. Glucksberg, K. G. Lerner	25:179-98
Clinical Utility of In Vitro Evaluation of Granulopoiesis	P. L. Greenberg, S. L. Schrier	25:269-78
Molecular Immunology of Factor VIII	T. S. Zimmerman, T. S. Edgington	25:303-14
Control of Platelet Production	L. A. Harker	25:383-400
Intravascular Coagulation in Liver Disease	M. Verstraete, J. Vermylen, D. Collen	25:447-55

IMMUNOLOGY

Immunologic Aspects of Renal Disease	C. B. Carpenter	21:1-16
Current Concepts of Interferon and Interferon Induction	E. De Clercq, T. C. Merigan	21:17-46
Antibody Variability	R. Pink, A.-C. Wang, H. H. Fudenberg	22:145-70
Neutrophil Proteases in Inflammation	A. Janoff	23:177-90
Interspecies Patterns of Slow Virus Diseases	R. W. Leader, A. I. Hurvitz	23:191-200
Immunologic Reconstitution	W. D. Biggar, B. H. Park, R. A. Good	24:135-44
Transfer Factor Therapy in Immune Deficiency States	A. S. Levin, L. E. Spitler,	

	H. H. Fudenberg	24:175-208
Animal Models of the Asthmatic State	R. Patterson, J. F. Kelly	25:53-68
Characterization of Amyloid	G. G. Glenner, W. D. Terry	25:131-35
Immunopathogenesis of Systemic Lupus Erythematosus	D. Koffler	25:149-64
INFECTIOUS DISEASE		
Mycoplasmas as Infectious Agents	L. Thomas	21:179-86
Human Reaginic Antibodies	K. Ishizaka	21:187-200
Toxoplasmosis: Recent Developments	J. S. Remington	21:201-18
Superinfection in Lymphoreticular Diseases	Z. A. McGee. W. Schaffner, M. G. Koenig	22:25-38
The Role of Australia Antigen in Viral Hepatitis and Other Diseases	A. I. Sutnick, I. Millman, W. T. London, B. S. Blumberg	23:161-76
Preventive Antibiotic Management in Surgery	J. F. Burke	24:289-94
Venezuelan Equine Encephalitis Virus Infection in Man	N. J. Ehrenkrantz, A. K. Ventura	25:9-14
Food Poisoning Due to Vibrio Parahaemolyticus	W. H. Barker Jr., E. J. Gangarosa	25:75-81
Preventive Therapy of Tuberculosis	D. L. Brummer	25:115-22
Infectious Complications of Nonalcoholic Drug Abuse	D. B. Louria	25:219-31
Antiviral Chemotherapy	J. P. Luby, M. T. Johnson, S. R. Jones	25:251-67
MISCELLANY		
Human Chromosome Damage by Chemical Agents	M. W. Shaw	21:409-32
Kinins in Human Disease	D. L. Wilhelm	22:63-84
Chromosome Identification	K. Hirschhorn	24:67-74
Genetic Aspects of Gout	M. A. Becker, J. E. Seegmiller	25:15-28
Medical Aspects of Recreational Skin and Scuba Diving	J. P. Sanford	25:401-10
MUSCULO-SKELETAL DISORDERS		
Crystal-Induced Inflammation of the Joints	D. J. McCarty	21:357-66
Myasthenia Gravis and the Thymus	G. Goldstein	22:119-24
Alcoholic Myopathy	G. T. Perkoff	22:125-32
Crystal Deposition Joint Disease	D. J. McCarty	25:279-88
NEPHROLOGY		
Renal Regulation of Sodium Excretion	I. H. Mills	21:75-98
Intraleukocytic Microbicidal Defects	S. J. Klebanoff	22:39-62
Hemodialysis in the Management of Renal Disease	W. J. Kolff	23:321-32
Renin Assay in Hypertensive Disease	J. Lowenstein	23:333-42
Urethrovesical Dysfunction and Infection	F. Hinman	24:83-88
Renal Energy Metabolism and Sodium Reabsorption	P. R. Lewy, A. Quintannila, N. W. Levin, R. H. Kessler	24:365-84
Immunopathology and Glomerulonephritis	C. B. Wilson, F. J. Dixon	25:83-98
NEUROLOGY		
Cerebral Lipidoses	R. O. Brady	21:317-34
Thermal Control in Premature Infants	J. C. Sinclair	23:129-48
Nutrition, Environment, and Behavioral Development	M. Winick, J. Coombs	23:149-60
The Lesch-Nyhan Syndrome	W. L. Nyhan	24:41-60
Metabolism of Ketone Bodies by the Brain	L. Sokoloff	24:271-80
NUTRITION		
Dietary Treatment of Inborn Errors of Metabolism	N. A. Holtzman	21:335-56
Inducible Metabolic Abnormalities During Development of Obesity	E. A. H. Sims, E. S. Horton, L. S. Salans	22:235-50
Modification of Brain Serotonin by the Diet	J. D. Fernstrom	25:1-8
ONCOLOGY		
Chromosomal Abnormalities in Human Neoplasia	A. A. Sandberg, D. K. Hossfeld	21:379-408
L-Asparaginase	R. L. Capizzi, J. R. Bertino,	

	R. E. Handschumacher	21:433-44
Antigenic Reversion in Human Cancer	P. Gold	22:85-94
Ablation Therapy for Hormone-Dependent Tumors	T. L. Dao	23:1-18
Immunity to Neuroblastomas and Melanomas	K. E. Hellström, I. Hellström	23:19-38
Epstein-Barr Virus Infection in Relation to Infectious Mononucleosis and Burkitt's Lymphoma	R. T. Smith, J. C. Bausher	23:39-56
Current Concepts in Treatment of Lymphomas with Special Reference to Hodgkin's Disease	L. Stutzman	24:325-34
Medical Management of the Hypercalcemia of Malignancy	L. J. Deftos, R. Neer	25:323-31
The Cell Surface and Malignant Transformation	R. E. Pollack, P. V. C. Hough	25:431-46
OPHTHALMOLOGY AND OTORHINOLARYNGOLOGY		
Senile Macular Degeneration	P. Henkind	22:95-102
Optic Atrophy and Neurosyphilis	J. L. Smith, C. W. Israel	22:103-18
PSYCHIATRY		
Chemotherapy of Mental Depression	L. J. King	21:367-78
Biogenic Amines and Behavior	R. J. Baldessarini	23:343-54
Adopted Children and Their Families in the Evaluation of Nature-Nurture Interactions in the Schizophrenic Disorders	P. H. Wender	23:355-72
Genetic Analysis of Human Behavior	B. Childs	23:373-406
Learned Helplessness	M. E. P. Seligman	23:407-12
Chemical Mutagens	M. S. Legator	23:413-28
Behavioral Aspects of Chromosomal Disorders	S. Kessler, R. H. Moos	24:89-102
Mental Effects of L-Dopa	D. L. Murphy	24:209-16
Pharmacotherapy of Schizophrenia	G. L. Klerman	25:199-217
Biogenic Amines and Affective Disorders	J. J. Schildkraut	25:333-48
PUBLIC HEALTH AND ENVIRONMENTAL MEDICINE		
Smallpox and Smallpox Vaccination Policy	J. M. Lane, J. D. Millar, J. M. Neff	22:251-72
Use of Physicians' Assistants in the Delivery of Medical Care	E. A. Stead Jr.	22:273-82
Methadone Treatment of Heroin Addiction	D. X Freedman, E. C. Senay	24:153-64
REPRODUCTIVE SYSTEM AND OBSTETRICS		
Fetal Indications for Therapeutic Abortion	A. C Barnes	22:133-44
The Prenatal Diagnosis of Inborn Errors of Metabolism	A. Milunsky, J. W. Littlefield	23:57-76
Estimation of Gestational Age in the Fetal State	J. W C. Johnson Jr., G. B. Odell	24:165-74
RESPIRATION		
Cystic Fibrosis in Adults	S. W. Brusilow	21:99-104
Alpha1 Antitrypsin Deficiency and Pulmonary Emphysema	C. A. Guenter, M. H. Welch, J. F. Hammarsten	22:283-92
Clinical Application of Continuous Monitoring of Respiratory Failure	K. F. Tantum, R. D. Dripps	22:293-304
Mechanics of Air Flow in Airway Obstruction	A. C. DeGraff Jr., A. Bouhuys	24:111-34
Endogenous Carbon Monoxide Metabolism	R. F. Coburn	24:241-50
THERAPEUTICS AND TOXICOLOGY		
Metabolic Modification of Parkinson's Disease and of Chronic Manganese Poisoning	G. C. Cotzias, P. S. Papavasiliou, J. Ginos, A. Steck, S. Düby	22:305-26
Effects of Oral Contraceptive Agents and Sex Steroids on Carbohydrate Metabolism	R. E. Kalkhoff	23:429-38
Lithium Salts in the Management of the Manic-Depressive Syndrome	S. Gershon	23:439-52
Pharmacogenetics: Defective Enzymes in Relation to Reactions to Drugs	B. N. La Du	23:453-68
Drug Metabolism in the Newborn	M. Eriksson, S. J. Yaffe	24:29-40
Clinical Pharmacology of Drug Interactions	I. H. Raisfeld	24:385-418
The Effects of Fluorinated Anesthetics on the Liver and Kidneys	L. S. Gottlieb, C. Trey	25:411-29